EXHIBIT 1

Exhibit 1
Glossary of Terms

Anion Exchange Chromatography: A separation method that employs a chromatography column that contains a positively charged solid material that binds negatively charged substances present within an antibody solution that is passed over the solid material, such that the negatively charged substances bound to the solid material are retained in the column, while allowing the remaining solution to pass through.

Antibody: A large, generally "Y"-shaped protein produced by the immune system in response to an antigen. The antibody specifically binds to the antigen and assists in its destruction.

Antibody/Antigen Complex: An antibody that is bound to its specific antigen.

Anticomplement Activity ("ACA"): The amount of protein capable of activating 50% of the complement in an optimally titered complement and red blood cell/hemolysin system, as determined by a particular anticomplement activity assay.

Antigen: A substance that stimulates an immune response, especially the production of antibodies. An antigen is usually a protein or polysaccharide, for example, a protein or polysaccharide on the surface of a bacteria or virus.

Biological Assay: An assay that involves use and/or measurement of cells, viruses, proteins or other molecules of biological origin. An example of a biological assay is an ACA assay that relies on red blood cell lysis. Reagents of biological origin inherently have more variability than, for example, industrial chemicals, which can result in an higher level of variability of biological assays than in chemical tests.

CH_{50} Unit: The amount of complement that results in lysis of 50% of a specified amount of optimally sensitized red blood cells as measured in an optimally-titered complement and red blood cell/hemolysin system.

Chromatography Column: A column containing a solid material, over which a solution is passed. Some components within the solution will be retained by the solid material, as a result of interactions between the solid material and the retained component, while the remaining solution and other components in the solution pass through the column. In some cases, the retained component will be the component of interest and will be later recovered from the solid material. In other cases, the component of interest will pass through the column, and the solid material will retain other components of the solution (such as contaminants).

Cohn-Oncley Method/Fractionation: A generic, modifiable method for separating human plasma into various components, *e.g.*, albumin, immunoglobulins and clotting factors.

Complement/Complement Protein: One of a number of proteins within the complement system which are part of an enzymatic cascade (*i.e.*, a series of protein-catalyzed chemical reactions, the products of which serve as the precursors for subsequent reactions within the

cascade, thereby catalyzing the subsequent reactions and enhancing the cascade) that can be triggered by association with an antibody complex (such as hemolysin sensitized sheep red blood cells or aggregates of antibodies).

Complement System: An enzymatic cascade of the immune system that, *inter alia*, attacks pathogens. The complement system comprises many plasma proteins that work together to, *inter alia*, lyse an invading pathogen by disrupting the pathogen's plasma membrane.

Hemolysin: An antibody used to sensitize red blood cells such that complement will bind to and lyse the red blood cells.

Immunoglobulin: See antibody.

Inactivate: To render a virus unable to infect a cell or otherwise replicate. In the context of virology, viruses can be rendered inactive by, for example, disrupting the membrane coating of the viruses, or otherwise preventing the viruses from infecting cells.

Incubation: Holding a solution (*e.g.*, an antibody solution) under particular conditions for a particular amount of time.

Ionic Strength: A quantification of ions in solution calculated as the summation $I = \frac{1}{2}\sum_i c_i z_i^2$ where c_i is the concentration of each type of ion (in moles/L) and z is its charge

Lipid Enveloped Virus: A virus surrounded or "enveloped" by a lipid-based membrane.

Lysis: The bursting of a cell, *e.g.*, by destroying the integrity of the cell membrane.

Nanofiltration: A process of filtration that physically prevents particles (such as large viruses) of a certain size from passing through the pores (holes) in a filter, while allowing molecules of interest in solution, such as antibodies, to pass through the pores, thereby separating out the larger unwanted particles.

Optimally Titered Complement and Red Blood Cell/Hemolysin System: The combination of complement, sheep red blood cells ("SRBCs") and hemolysin, each in amounts specific for the system such that a decrease of complement (*e.g.*, when mixed with an antibody test solution having ACA) can be measured as a change in the amount of SRBC lysis.

Plasma: The liquid portion of blood, from which blood cells have been removed. Plasma comprises various components, *e.g.*, albumin, immunoglobulins and clotting factors.

Release Criteria: Parameters (including ranges and limits) set and approved by regulatory agencies, such as the U.S. Food and Drug Administration, which a licensed product must meet before being released for sale.

Sensitized Sheep Red Blood Cells ("sSRBCs"): Red blood cells from sheep that have been treated ("sensitized") with hemolysin (the hemolysin attaches to the surface of the cell, making the red blood cells capable of being lysed).

Serum: The blood plasma from which the clotting factors have been removed.

Solvent/Detergent: A mixture of a solvent (typically a trialkylphosphate) and a detergent (a cleaning agent such as sodium cholate, polysorbate (Tween-80), and octoxynol (Triton X-100).

Trialkylphosphate: Solvents used in solvent/detergent treatment are typically trialkylphosphates, the most well known being tri(n-butyl)phosphate or "TNBP."

EXHIBIT 2

US006686191B1

(12) **United States Patent**
Alonso

(10) Patent No.: US 6,686,191 B1
(45) Date of Patent: Feb. 3, 2004

(54) **PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1772 days.

(21) Appl. No.: 08/532,231

(22) Filed: Sep. 22, 1995

(51) Int. Cl.[7] C12N 7/04; A61K 39/395; A61K 39/40; A61K 39/42

(52) U.S. Cl. 435/236; 424/176.1; 424/177.1; 424/130.1

(58) Field of Search 530/390.1, 390.5, 530/386, 387.1; 424/176.1, 177.1, 130.1; 435/236

(56) References Cited

U.S. PATENT DOCUMENTS

4,396,608 A * 8/1983 Tenold
4,540,573 A * 9/1985 Neurath et al.
4,762,714 A * 8/1988 Mitra et al.

OTHER PUBLICATIONS

Joy Yang, Y.H. et al. "Antibody Fc functional activity of intravenous immunoglobulin preparations treated with solvent-detergent for virus inactivation" Vox Sang, vol. 67, pp. 337-344, May 17, 1994).*

* cited by examiner

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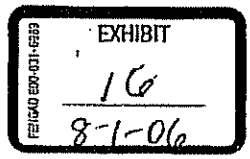
(57) **ABSTRACT**

Method of reducing the anticomplement activity (ACA) resulting from viral inactivation treatment of a solution of antibodies, the method comprising contacting the solution with a trialkylphosphate, such as tri-n-butyl phosphate, and a detergent, such as sodium cholate, under conditions sufficient to reduce substantially the virus activity, and then incubating the solution under controlled conditions of time, pH, temperature, and ionic strength such that the anticomplement activity is reduced to an acceptable level. In a preferred embodiment, the ACA is reduced to less than 60 CH_{50} units/mL, the incubation is for at least about ten days at a pH from 3.5 to 5.0, the temperature is maintained within a range of 2 to 50° C., and the ionic strength of the solution is less than about 0.001 M.

24 Claims, 1 Drawing Sheet



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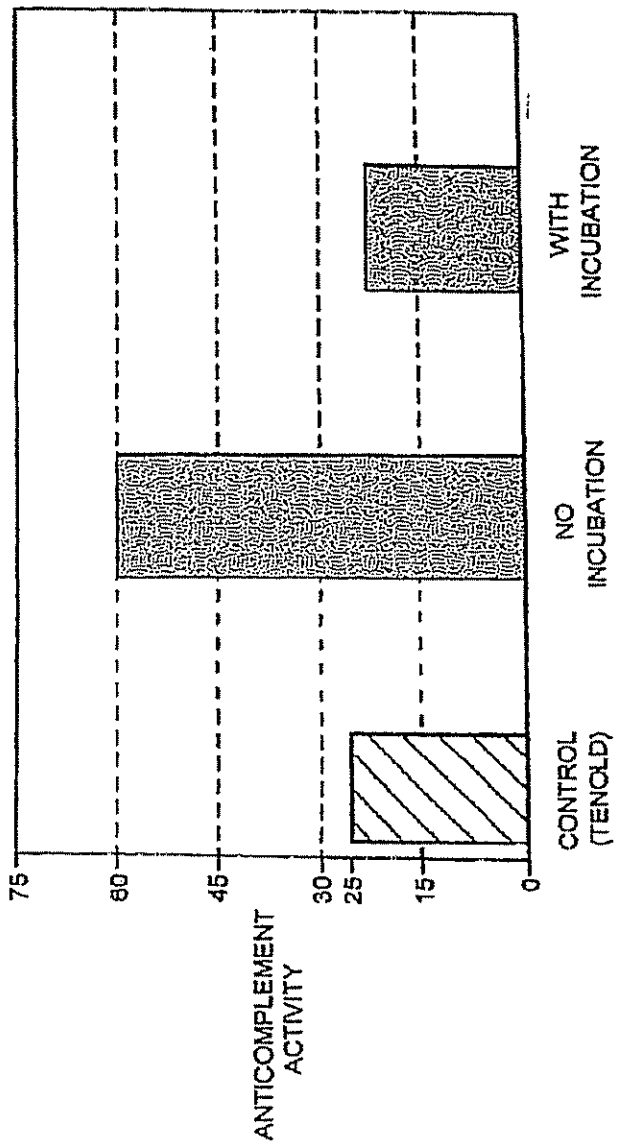
75
60
45
30
25
15
0
ANTICOMPLEMENT ACTIVITY
CONTROL (TENOLD)
NO INCUBATION
WITH INCUBATION

PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

BACKGROUND OF THE INVENTION

1 Field

This invention generally deals with an intravenously injectable immunoglobulin product, and more specifically deals with an intravenously injectable immune serum globulin (IGIV) which has been subjected to a virus inactivation step and which has a low level of anticomplement activity.

2. Background

Early pharmaceutical preparations of immune serum globulins could not be administered intravenously due to an unacceptably high incidence of adverse reactions. These adverse reactions were associated with a decrease in serum complement levels, apparently caused by complement binding to the administered gamma globulin. (1) The ability of gamma globulin to bind complement, or its anticomplement activity (ACA), is greatly increased as a result of denaturation brought about during the fractionation procedure Several approaches have been taken to address the problem of rendering ISG safe for intravenous administration. (See (2) and references therein). Tenold reported a method of preparing an immune serum globulin (ISG) with low ACA which could be administered by intravenous injection. (2, incorporated herein by reference). The Tenold '608 process requires formulating the ISG at low ionic strength (preferably less than about 0.001) and at low pH (3.5–5.0).

Other methods of preparing intravenously injectable immune serum globulin (IGIV) have been reported, including stabilizing with carbohydrates such as maltose (3). A process including incubation of ISG at pH 4.0 at 37° C. (4) results in a product with low ACA which may be administered by intravenous injection; however, upon storage the product regains its high ACA. IGIV has also been prepared by covalent modification of the ISG, for example by proteolysis (5) or by reduction of disulfide linkages followed by reaction with a blocking agent (1,6).

Antibody preparations, since they are isolated blood products, have an inherent hazard of transmitting virally-mediated diseases. Inactivation of viruses is an important step in producing safe and effective blood products. U.S. Pat. No. 4,540,573 to Neurath et al., which is incorporated herein by reference, describes a viral inactivation process using a trialkyl phosphate and detergent process (hereinafter, the solvent/detergent process, or SD process). (7) That solvent/detergent method has gained acceptance as being efficacious in the inactivation of lipid-enveloped viruses with limited adverse effects on biological activity or blood product profile. (8, 15; See also 12 for a discussion of various viral inactivation processes)

Current antibody preparations on the market generally have been regarded as safe with respect to viral contamination. (9) This is thought to be due to features of the fractionation processes used to isolate these blood products. However, it would be desirable to further ensure the safety of the antibody preparations by including a distinct viral inactivation step in the production process Successful reduction of viral activity in an IGIV solution was reported using several different methods of viral inactivation for a variety of viruses (16, 17) A process for preparation of immunoglobulins substantially free of retrovirus has been reported involving incubation of ISG under controlled conditions of time, temperature, and pH. The process entails isolating ISG via a cold ethanol plasma fractionation process and then storage of the ISG at one of two storage conditions: (a) at pH≦4.25 at a temperature of 27° C. for at least three days, or (b) at pH≦6.8 at a temperature of 45° C. for at least six hours. (10).

We have found that using the SD process to treat ISG preparations, especially those subsequently formulated according to the Tenold '608 patent, results in a product with an acceptable viral inactivation but with unacceptably high levels of ACA. Elevated ACA levels were always detected at the sterile bulk stage (i.e., after compounding to 5% or 10% IGIV and filtration with 0.2 μm sterile filters) of all tri-n-butyl phosphate (TNBP)/detergent treated IGIV preparations regardless of process scale. Preparations of ISG with high ACA levels are not suitable for intravenous injection and instead must be administered via other routes, e.g. intramuscular (IM) injection. However, IGIV preparations are more desirable since they are immediately available in the bloodstream and are not subject to loss associated with IM injection. It is thus desirable to have an IGIV product which is both low in ACA and has been subjected to a viral inactivation step.

SUMMARY OF THE INVENTION

The invention is a method for producing an intravenously injectable immune serum globulin (IGIV) preparation with low anticomplement activity which has been chemically treated to render it substantially free of lipid-enveloped viruses. The method comprises a solvent/detergent viral inactivation step followed by an incubation step. We have discovered that the incubation step is necessary to achieve an acceptable level of ACA low enough to allow the ISG to be administered by intravenous injection. The incubation step should be conducted under controlled time, pH, temperature, and ionic strength Preferably, the pH should be maintained between about 3.5 and about 5.0, the temperature should be within a range of about 2 to about 50° C., and the ionic strength should be less than about 0.001M. In a preferred embodiment the ACA of the ISG preparation decreases gradually over a period of at least about ten days when the ISG is maintained at a pH of about 4.25 at low ionic strength (less than about 0.001M) and the viral inactivation step (in a model system) results in a substantial reduction (i.e. at least 4 logs) in the titer of lipid enveloped viruses.

BRIEF DESCRIPTION OF THE FIGURE

FIG 1 shows a comparison of the typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation of the present invention.

SPECIFIC EMBODIMENTS

Materials and Methods

The starting material for the process of this invention is unmodified human immune serum globulin. In the specification and claims the term "immune serum globulin" is used to define the substance also referred to in the literature variously as gamma globulin, IgG and immunoglobulin G. It consists predominantly and preferably of at least about 85 percent of the 7S species of gamma globulin, which has a molecular weight of about 160,000 Any remainder is preferably 9S species, with a molecular weight of about 300,000. Both standard immune and hyperimmune serum globulins, e.g., tetanus, rabies and hepatitis immune serum globulins,

can be employed, the solvent/detergent treated product being immune and hyperimmune ISG respectively. Thus, a suitable starting material for the process of this invention is Cohn's Fraction II or Fraction III filtrate. (See Refs 13, 14.)

Fraction II, by ultracentrifugation studies, is predominantly (about 85 percent) the 7S (sedimentation constant of 7) species of gamma globulin with an average molecular weight of 160,000. The remaining protein is essentially 9S material with a M.W. of about 300,000. Wet Fraction II paste (approximately 30 percent solids) is commonly lyophilized to obtain dry ISG powder which is then dissolved and prepared for intramuscular injection as a 16.5 percent sterile solution. Either the wet Fraction II paste or the dry ISG powder is a suitable starting material for the process of this invention.

Gamma globulin obtained by any process which has essentially the same composition of protein components as found in the Cohn Fraction II or Fraction III filtrate can be used as starting material in the present process. Both standard immune serum globulin and hyperimmune serum globulin can be employed as starting materials. As is well known, the latter is produced from plasma or serum obtained from selected donors who have much higher titers for a specific antibody than is normally found in the average population. These donors have either been recently immunized with a particular vaccine or else they have recently recovered from an infection or disease. These high titer sera or plasmas are pooled and subjected to the usual Cohn fractionation procedures up to the point of isolating Fraction II.

Furthermore, because the amount of antibody required to achieve a desired immunological response is substantially less when administered intravenously, it will be apparent the intravenous dose will be substantially less than the intramuscular dose which will produce the same serum antibody titer. Thus, the dose of intramuscular ISG and hyperimmune serum globulin must be higher than that required to achieve the same serum antibody titer when globulin of the same antibody activity is administered intravenously.

The starting wet paste or lyophilized powder is dissolved in a volume of water or other physiologically-acceptable carrier to provide a protein solution of a concentration of about 0.5–20% preferably about 5 to 10 percent. If Fraction III filtrate is employed, the aqueous solution must be concentrated by conventional techniques to the desired protein concentration. Any protein concentration may be used in this method; however, the above range is preferred from a practical standpoint.

After the protein has been dissolved or concentrated, the solution is adjusted to a pH of about 3.5 to 5.0 preferably about 3.8 to 4.2, by addition of a physiologically-acceptable acid such as hydrochloric acid. In general, the pH is adjusted to a point whereat the monomeric material in the protein solution is maintained at a maximum. However, the pH must not be so low as to result in gelation. The temperature should not be harmful to the ISG material. Good results are obtained within the temperature range of about 0–20° C. It is not necessary to hold the so-adjusted material for any period of time prior to the next step; however, the material may be held, if desired, without detrimental effects.

The protein solution at the appropriate pH (preferably 3.8–4.2) may be diafiltered with at least 4 volume exchanges of water to reduce the alcohol concentration from approximately 17% (Filtrate III) to about 2% alcohol. The efficacy of solvent/detergent as a viral inactivation method is much better at or above ambient temperatures; however, high concentrations of alcohol at these temperatures will denature the IgG molecules. Thus, this inactivation must be performed in low alcohol concentration.

Next, the protein concentration of the so-treated material is adjusted to the level desired for incubation with TNBP/detergent, generally less than 10% protein for maximum viral inactivation. This adjustment is accomplished by conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Prior to addition of TNBP/detergent, the pH may be adjusted within a wide range, depending on the detergent to be used. With Tween 80, the pH may be as low as 3.5, where the IgG starts becoming unstable. With cholate, the pH is adjusted to within the range of 5.0–6.4, preferably about 5.6, prior to addition of TNBP/detergent. Satisfactory cholate solubility during incubation was achieved by adjusting the immunoglobulin solutions to a pH of 5.5 or higher prior to addition of TNBP and sodium cholate. Adjusting the IgG solution to pH values lower than 5.5 is not suitable because the solubility of sodium cholate is highly dependent on pH (cholic acid pK=6.4), with poor solubility at pH 5.5 or lower. Furthermore, maximum viral inactivation during incubation with TNBP/cholate was observed at pH values less than 6.0 in experiments which employed model viruses spiked into IgG solutions. The inactivation of HIV-1 and BVDV (bovine viral diarrhea virus, which is employed as a model for hepatitis C) was accelerated at pH 5.8, with inactivation to the detection limit occurring in 1–2 hours, whereas inactivation to the detection limit required a minimum of 6 hours when pH 7 conditions were used.

Next, the TNBP/detergent is added to the protein solution (preferably less than 8% (w/w), pH 5.8) mixed thoroughly, and then incubated above ambient temperatures, for example 30° C., with continuous agitation or mixing. Target TNBP/cholate levels for optimal viral inactivation during the incubation step should be >3 mg/mL TNBP and >2 mg/mL cholate as defined by Edwards et al (8) Moreover, for effective viral inactivation, it is important that the solution is essentially free of particulates in order to facilitate thorough mixing of solvent/detergent and IgG solution. After incubation with TNBP/cholate under these conditions, greater than 5.2 $\log_{10}$ reduction of HIV-1 and greater than 4.0 $\log_{10}$ reduction of BVDV were detected.

After completing the incubation which provides the viral inactivation, the solvent and detergent molecules must be removed in order to achieve a final product with low levels of residual TNBP and cholate which would be suitable for intravenous administration. Generally, procedures to remove detergent are also effective in removing TNBP, and vice versa. Very low levels of TNBP and cholate in the final container can be achieved by a combination of filtration, diafiltration and hydrophobic chromatography. After completing the incubation, the majority of cholate (and TNBP) can be removed from the protein solution by filtration, providing the solution had been previously adjusted to a lower pH value such as 4.0, because sodium cholate is sparingly soluble in aqueous solutions at such pH values. Moreover, all processing steps which follow the solvent/detergent incubation are performed at lower pH values (i.e., 4.0) because IgG molecules are more stable at pH values between 3.5–5.0, in low ionic strength solutions (2) Thus, after incubation with TNBP/cholate, the protein solution is adjusted to approximately pH 4.0 and incubated at 0–8° C. in order to promote cholate precipitation. Next, filtration is employed to remove the precipitated cholate from the IgG solution.

The so-treated solution is diafiltered with at least four volume exchanges of water to reduce the ionic strength and

to remove additional TNBP and cholate. After or during the above treatment, the pH is measured and maintained within the range of about 3.5–5.0. The protein concentration of the so-treated material is adjusted to 10–30%, usually 13% (w/v) by employing conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Again the pH of the preparation is maintained within the range of about 3.5–5.0, preferably about 3.8–4.2.

In the present invention, hydrophobic chromatography is employed to remove the TNBP and cholate not eliminated by the filtration and diafiltration steps, and thus provide a final product with low levels of residual TNBP and cholate which is suitable for intravenous administration. Hydrophobic chromatography is a method for TNBP removal from protein solutions that has fewer drawbacks and limitations than other available methods such as oil extraction, ion exchange or affinity chromatography. In part, this is because the protein of interest (IgG) remains in solution throughout the TNBP removal process. Polystyrene-based resins (typically PLRP-S from Polymer Laboratories, Amherst, Mass.) were used to remove the solvent/detergent from solution, as we have found the polystyrene-based resins to be superior to other resins, such as silica-based C-18 resins.

Next, the ISG preparation is adjusted to 5% or 10% protein, and treated to render it ionic i.e., to render it compatible with physiological conditions, or render it physiologically acceptable upon injection. In a preferred embodiment, the tonicity is adjusted to about 230 to about 490 mosmol/kg solvent. More preferably, the tonicity range is from about 250 to about 350 mosmol/kg solvent, and most preferably the tonicity range is from about 260 to about 325 mosmol/kg solvent. The 5% formulation (5% IGIV) is made tonic by the addition of 10% maltose. The 10% formulation contains 0.2 M glycine in order to achieve an isotonic preparation without large quantities of sugar. The product with either formulation (Gamimune®N 5% or Gamimune®N 10%) experiences shifts in molecular distribution (antibody aggregation) when the ionic strength of the low pH solution is increased. Therefore, sodium chloride, which is often used to achieve tonicity, should not be used.

The so-treated solution is incubated at pH 4.25 under low ionic strength conditions (NLT 21 days at 20–27° C. preferred) in order to provide a lowering of ACA levels. The ionic strength is determined according to Perrin (18), and in a preferred embodiment the ionic strength should be less than about 0.001M. Elevated ACA levels were always detected at this stage of all TNBP/cholate treated IGIV preparations (regardless of process scale); however, ACA levels are gradually lowered by incubation at pH 4.25 under low ionic strength conditions (Tables 3, 5–7). While there is no strict rule for determining when the ACA level is low enough to be an acceptable level suitable for intravenous administration, IGIV preparations should have ACA levels as low as possible.

The Figure depicts the typical average reduction of ACA observed in 5% IGIV solutions following SD treatment. For a 5% ISG formulation the acceptable level suitable for intravenous administration preferably would be less than about 45 CH_{50} units/mL, and more preferably less than about 30 CH_{50} units/mL. For a 10% ISG formulation, the acceptable level suitable for intravenous administration preferably would be less than about 60 CH_{50} units/mL, and more preferably less than about 45 CH_{50} units/mL. As used herein, one unit of ACA activity (one CH_{50} unit) is defined as the amount of protein capable of activating 50% of the complement in an optimally titered complement and red blood cell/hemolysin system. The assay measures the amount of complement that is bound by the mixture of standardized amounts of complement and protein. See refs. 19–20 for a discussion of the assay. Briefly, red blood cells that have been sensitized by preincubation with red blood cell antibodies are added to the complement/protein mixture. In the presence of free complement (not already bound by the protein) these sensitized cells will lyse, releasing hemoglobin which can be quantitated as a measure of the degree of lysis. In parallel, sensitized red blood cells are also added to a buffer control-complement mixture, whose degree of lysis is defined as 100%. The difference between the actual amount of complement needed to give 100% lysis and the amount of complement remaining unbound in the presence of protein equals the amount of complement actually bound by the protein, or anticomplement activity.

Results

Anticomplement Activity of ISG Resulting From Viral Inactivation Process

To establish the effect of the SD viral inactivation process on solutions containing ISG which are formulated according to the Tenold '608 patent, the experiments depicted in Table 1 were performed. The starting material (SM) was Cohn process filtrate III which had been ultrafiltered to about 5% protein and then diafiltered with four volumes of water.

In the control experiment, incubation (−)/SD (−), the SM was not subjected to any incubation or solvent/detergent treatment. In the incubation (+)/SD (−) experiment, the pH of the SM was adjusted to 7.0, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/SD, TNBP & Tween 80 (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL Tween 80 were added to the solution, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/SD, TNBP & cholate (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL cholate were added to the solution, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. The solutions in each experiment were then diafiltered with four volumes CWFI (cold water for injection) and concentrated by ultrafiltration. After addition of dry maltose to 10% w/v, the 5% IGIV solution (pH 4.25) was filtered through a 0.2 μm filter.

TABLE 1

Anticomplement activity in 5% IGIV produced by variations of the Solvent/Detergent IGIV Process

	ACA (CH_{50}/mL)
Control (no solvent/detergent treatment, no 30° C. incubation)	25
Incubate at 30° C. for 10 hr (no solvent/detergent)	22
Incubate at 30° C. for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL Tween 80	68
Incubate at 30° C. for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL cholate	>100

*These samples were assayed for ACA after final compounding according to the Tenold '608 patent, but they were not incubated at pH 4.25 and 27° C. prior to assay.

The results listed in Table 1 show that levels of ACA increased in IgG samples after incubation with TNBP/cholate or TNBP/Tween 80. ACA levels were not elevated in IgG samples that were incubated for 10 hr at 30° C. in the

absence of solvent/detergent. These results suggest that ACA levels of IGIV samples were not elevated by either processing manipulations or incubation for 10 hr at 30° C. in the absence of solvent/detergent.

TABLE 2

Anticomplement activity in 5% IGIV spiked with TNBP/Na cholate

	ACA (CH_{50}/mL)
5% IGIV, no TNBP/cholate	12
5% IGIV with 100 μg/mL TNBP, 100 μg/mL Na cholate	13

Furthermore, spiking experiments (with TNBP and Na cholate, Table 2) have demonstrated that the elevated anticomplement activity levels were not artifacts caused by carrying out the anticomplement assay in the presence of trace levels of TNBP/Na cholate. Thus, using the prior art SD process for viral inactivation of a solution containing ISG, subsequently formulated according to the Tenold '608 patent, yields a product which has high ACA and is unsuitable for intravenous administration. In a similar experiment, SD treated samples which were not incubated (Table 3, Initial Testing) had ACA levels greater than 100 units.

TABLE 3

Reduction in Anticomplement activity of samples previously treated with TNBP/cholate

Sample	ACA (CH_{50}/mL) Initial Testing (no incubation)	ACA (CH_{50}/mL) After incubation 6 wk. @ 5° C. 3 wk. @ 22° C.
RD21872-16	>100	33
RD21872-17	>100	34
RD21872-18	>100	26
RD21872-20	>100	27

However, when duplicate SD treated samples were incubated for extended periods of time (6 weeks at 5° C. and 3 weeks at 22° C.), the level of ACA was markedly reduced (Table 3, after incubation). This led to further investigation of this surprising observation.

Aggregate Content of ISG Exposed to TNBP/cholate

The samples of the previous experiment (Table 3, Initial Testing) were analyzed by size exclusion (gel permeation) HPLC immediately after compounding to determine the extent of aggregation of the IGIV at the initial time point. HPLC analysis shows nearly complete monomer content in the samples. (Table 4).

TABLE 4

HPLC analysis of non-incubated 5% IGIV samples (Table 3 Initial)

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
RD21872-16, Initial	0.140	0.00	99.86	0.00
RD21872-17 Initial	0.146	0.00	99.85	0.00
RD21872-18 Initial	0.124	0.00	99.88	0.00
RD21872-20, Initial	0.172	0.00	99.83	0.00

Previously, high IgG aggregate levels were shown to correlate with high anticomplement activity. However, results from analysis of the samples show the level of ACA in the samples to be greater than 100 units. (Table 3, 'Initial Testing') The HPLC analysis shows that the high ACA following the TNBP/cholate treatment was not due to the presence of aggregated IgG molecules.

Varied Conditions of Time and Temperature

The SM was the same as in the previous experiment, and experimental conditions were similar with the following changes. The solutions were treated with TNBP/cholate at pH 7.0 and then were compounded to 5% IGIV, 10% maltose, pH 4.25, as above. The ACA was assayed immediately after final compounding, after a first incubation for nine days at 5° C., and after a second incubation for 21 days at either 22° C. or 5° C. The results are presented in Table 5.

TABLE 5

ACA of TNBP/cholate treated IGIV samples

Sample Point	ACA (CH_{50}/mL)
Intermediate Samples	
Initial sterile bulk	>100
Incubated 9 d. @ 5° C.	>100
Final Incubation	
21 d. @ 22° C.	49
21 d. @ 5° C.	71

In the initial sterile bulk sample, which was treated with TNBP/cholate at pH 7.0, the level of ACA was again greater than 100 units for the initial time point, confirming the observations noted in Table 3. Upon incubation at 5° C. for nine days, the ACA remained greater than 100 units. The final incubation step at either 5° C. or 22° C. shows that the reduction in ACA is dependent on temperature, with faster reduction in ACA observed at higher temperatures.

Effect of pH During Solvent/detergent Treatment on ACA

ACA levels were evaluated after incubation with TNBP/cholate at pH 5.8 because better viricidal activity was observed at pH values less than 6.0. Generally, the non-incubated sterile bulk samples of material incubated at pH 5.8 had lower ACA levels than the pH 7.0 samples, but the trend of lowering ACA upon incubation was repeated in the pH 5.8 samples. In fact, the ACA levels continue to decrease beyond the 21 day incubation in samples that initially had elevated ACA levels after incubation with TNBP/cholate at pH 5.8 (Table 6). As was previously noted for the samples incubated at pH 7.0, the lowering of ACA was not due to decreasing levels of aggregated IgG molecules because the material treated at pH 5.8 was essentially monomeric IgG prior to 22° C. incubation (HPLC analysis, sample A4, Table 8).

TABLE 6

Sample A4 - ACA upon extended incubation

Incubation @ 22° C. (days)	CH_{50}/mL
0	122
10	73
19	55
25	56
28	45
30	40
34	39
41	33

TABLE 6-continued

Sample A4 - ACA upon extended incubation

Incubation at 22° C. (days)	CH_{50}/mL
48	30
55	29

Similar results were achieved with samples formulated to 10% IGIV, 0.2 M glycine in the sterile bulk stage. Upon incubation at low ionic strength at pH 4.25 for 10 and 21 days, the levels of ACA were seen to decline in both 5% IGIV samples and 10% IGIV samples. (Table 7) The decrease in ACA can thus be observed over a range of ISG concentrations and over a range of pH values for the solvent/detergent treatment. (Tables 3, 5, 7) HPLC analysis (Table 8) of the sterile bulk samples presented in Table 7 confirmed that the elevated ACA levels were not due to aggregation of ISG molecules

TABLE 7

ACA of samples treated with TNBP/cholate at pH 5.8

Sample	Sterile bulk (day zero) (CH_{50}/mL)	10 days incubation at 20-27° C. (CH_{50}/mL)	21 days incubation at 20-27° C. (CH_{50}/mL)
A1 (5% IGIV)	43	ND	10
A2 (5% IGIV)	31	14	15
A3 (5% IGIV)	44	15	12
A4 (5% IGIV)	122	73	55
B1 (10% IGIV)	>100	48	46
B2 (10% IGIV)	49	36	30
B3 (10% IGIV)	53	ND	37

Taken together, the above results suggest that ISG products which have been subjected to a solvent/detergent viral inactivation process resulting in an undesirable ACA increase can be made suitable for IV administration by incorporating an additional incubation step under the conditions described here to reduce the ACA to an acceptable level.

TABLE 8

HPLC Analysis of sterile bulk samples treated with TNBP/cholate at pH 5.8

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
A2	0.140	0.00	99.86	0.00
A3	0.146	0.00	99.85	0.00
A4	0.124	0.00	99.88	0.00

CONCLUSION

The ACA increase resulting from the solvent/detergent treatment of the IGIV (antibody) solution appears to be an unavoidable secondary effect of TNBP/detergent treatment to inactivate viruses in the solution. I have discovered that by incubating the solution of IGIV at low pH (4.25) and low ionic strength (0.001M) for a relatively long period of time (at least about 10 days), the ACA gradually decreases over the period of incubation.

The prior art discloses a method of producing IGIV (the Tenold '608 patent) using low pH and low ionic strength. The Tenold '608 method omits the viral inactivation step, and thus avoids the problem of increased ACA, but the possibility of viral activity remains. Unlike Tenold, incubation is an essential aspect of the present invention for reducing the ACA.

The Neurath et al. 573 patent teaches the solvent/detergent viral inactivation step. However, Neurath '573 does not mention controlling the pH and also does not mention any consequences of the process relating to ACA. Elevated ACA levels were detected at the sterile bulk stage of TNBP/cholate treated IGIV preparations. However, ACA levels decreased upon incubation for at least about 10 days at pH 4.25, low ionic strength, and not less than about 20° C. (See Tables 5–7) The prior art describes several approaches to lowering ACA levels of purified IgG preparations, including removal of IgG aggregates (11) IgG aggregates have been shown to activate the complement system in vivo. (1) In the present invention, however, lowering of IgG ACA was not due to decreasing levels of IgG aggregates because these TNBP/cholate treated IGIV preparations contained low levels of aggregated IgG (as measured by HPLC. Tables 4, 8) prior to incubation under such conditions.

It would be desirable to produce substantially virus-free IGIV, but following the prior art results in a product with an unacceptable level of ACA. Note that Tenold '608 states that the product is substantially free of ACA. but use of the SD process in conjunction with Tenold '608 does result in high levels of ACA; experimental results reported here show that treating ISG solutions with the SD process and then formulation according to the Tenold '608 patent leads to a product with high ACA. (See Tables 1, 3, 5–7) The surprising finding reported here is that a follow-up (terminal) incubation step lowers the ACA of the solvent/detergent treated solution. The typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation are compared in the Figure. The present invention thus includes a previously unobserved method of reducing the ACA by incubating under controlled conditions of pH, temperature, and ionic strength for a period of time, thus allowing the product to be administered by intravenous injection.

Mitra '714 does not suggest the use of a S/D process but, instead, reports that a relatively brief incubation of an ISG product under similar conditions results in a substantially virus free preparation (10) However, employing incubation under such conditions to provide a lowering of anticomplement activity is a novel application of these incubation conditions which were previously employed in the IGIV process for inactivation of enveloped viruses.

The newly developed IGIV process reported here, which includes an additional internationally accepted viral inactivation procedure (treatment with TNBP/cholate), generates IgG preparations which have low ACA levels and are suitable for IV administration. The major advantage is that an IGIV product with improved safety can be obtained by a two-step process that includes a TNBP/cholate treatment for viral inactivation and incubation under conditions that afford low ACA levels that are suitable for IV administration.

The above disclosure is intended to illustrate the invention, and it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention should be limited only by the claims below.

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What is claimed is:

1. A method of treating a solution of antibodies which may have virus activity, the method comprising

a) contacting the solution with a trialkylphosphate and a detergent under conditions sufficient to substantially reduce any virus activity and resulting in an increased level of anticomplement activity; and

b) then incubating the solution of step a) under conditions of controlled time, pH, temperature, and ionic strength, such that the increased anticomplement activity of the solution is reduced to an acceptable level suitable for intravenous administration.

2. The method of claim 1, wherein the anticomplement activity is reduced to less than about 60 CH_{50} units/mL.

3. The method of claim 1, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

4. The method of claim 3, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 30 CH_{50} units/mL.

5. The method of claim 1, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 60 CH_{50} units/mL.

6. The method of claim 5, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

7. The method of claim 1, wherein the incubation is for at least about ten days.

8. The method of claim 1, wherein the pH is maintained within a range of about 3.5 to about 5.0.

9. The method of claim 1, wherein the temperature is maintained within a range of 2° C. to 50° C.

10. The method of claim 1, wherein the ionic strength is less than about 0.001 M.

11. The method of claim 1, wherein at least about 99% of the antibodies are monomeric.

12. The method of claim 1, comprising the further step of adjusting the tonicity of the solution to a physiologic value under such conditions that the ionic strength is not appreciably altered.

13. The method of claim 12, wherein the tonicity of the solution is adjusted by adding a carbohydrate to the solution.

14. The method of claim 13, wherein the carbohydrate used is maltose.

15. The method of claim 12, wherein the tonicity of the solution is adjusted to a range of about 230 to about 490 mosmol/kg solvent.

16. The method of claim 15, wherein the tonicity of the solution is adjusted to a range of about 274 to about 309 mosmol/kg solvent.

17. The method of claim 12, wherein the tonicity of the solution is adjusted by adding an amino acid to the solution.

18. The method of claim 17, wherein the amino acid used is glycine.

19. The method of claim 1, wherein the trialkylphosphate is tri-n butyl phosphate and the detergent is selected from polysorbate 80 and sodium cholate.

20. The method of claim 1, wherein the solution has a pH between about 3.5 and about 6.0 during step a).

21. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001 M, a pH between about 3.5 and about 5.0, an antibody concentration of about 5% wt./wt., and a maltose concentration of about 10% wt./wt.

22. The preparation of claim 21, wherein the pH is about 4.25.

23. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001, a pH between about 3.5 and about 5.0, an antibody concentration of about 10% wt./wt., and a glycine concentration of about 0.2 M.

24. The preparation of claim 23, wherein the pH is about 4.25.

* * * * *

EXHIBIT 3

BLOOD SEPARATION AND PLASMA FRACTIONATION

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Antibody Purification From Plasma

RALPH H. ROUSELL and JOHN P. MCCUE

HISTORY OF IMMUNE GLOBULIN USAGE

Therapeutic application of immune globulin is dependent on two key elements: demonstration that administration of immune globulin concentrates is of therapeutic value and the ability to make sufficient quantities of pure and safe immune globulins.

Therapy With Immune Globulins

The initiation of immune globulin therapy may be attributed to Behring and Kitasato in Germany at the end of the last century. In 1890 they described the use of equine antitoxin raised against diphtheria toxin (Behring and Kitasato, 1890). This was followed in 1893 by the dramatic description of the successful treatment of a young girl terminally ill with diphtheria (Behring, 1893). Emil Behring was subsequently awarded the first Nobel Prize in Medicine in 1902 for this work and as recognition of the title conferred upon him became von Behring. Work on passive immunization against rabies using canine antisera was also underway at about this time in France (Babes and Lepp, 1889).

Human convalescent sera were first used in the early 1900s for the prevention of measles (Cenci, 1907), and later for pertussis (Debrê, 1923), mumps (Regan, 1925), and poliomyelitis (Bodian, 1949, 1951).

In the United States, work was proceeding as well. Maxwell Finland in 1930 reported his clinical experiences using equine serum to treat pneumococcal pneumonia at Boston City Hospital during the period 1919 through 1929. Over this period he and his co-workers modified the preparations to obtain more concentrated antibodies. As might be expected with the use of equine serum, febrile reactions were not uncommon and there was a significant frequency of serum sickness. In spite of these problems, there was a definite impression that serum therapy had resulted in improved survival rates in patients with pneumonia, which at that time was a disease with an extremely high mortality.

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Influence of the Sulfonamides and Antibiotics

Although work continued (Finland, 1936) using specific antiserum, by the mid-1930s sulfonamides were introduced (Domagk, 1935; Buttle et al., 1936; Colebrook and Kenny, 1936; Long and Bliss, 1939) and they were replacing serum therapy in the treatment of bacterial infections. The discovery of penicillin by Fleming in the early 1940s (Fleming, 1946) virtually ended the use of animal antisera to treat bacterial infections. The use of equine antisera against tetanus and diphtheria toxins and against the rabies virus continued even in the advanced countries well into the 1950s. Today the equine antisera are still used only in certain third world countries.

World War II and Plasma Fractionation

The start of the Second World War in Europe in 1939 alerted the medical advisors of the United States Armed Forces to the need for adequate supplies of whole blood and/or blood substitutes. In 1940 they began to gather in Washington (DeGowin and Hardin, 1941). As a result of the ongoing discussions and conferences, it soon became obvious that the most useful fraction obtainable from human plasma to resuscitate the severely injured and shocked individual would be albumin. Not only is it present in the largest amount in plasma, but it also has the greatest colloid osmotic efficiency, and is the most soluble, least viscous, and most stable of the plasma proteins. The first experiments on the fractionation of human plasma were conducted in August 1940 at the Harvard Medical School using plasma purchased from a few professional donors (Cohn, 1948). The actual details of the fractionation processes used have been described by Cohn et al. (1944, 1946). Thus while the prime intention of the experiments on fractionation of human plasma was to isolate albumin because of its value in resuscitating injured battle casualties, a spin-off of the work performed at Harvard was the concentration of the immune globulins in Cohn Fraction II + III.

Primary Immune Deficiency Syndromes

The basic therapeutic usage of immune globulin concentrates became firmly established in 1952 when Bruton published his classic description of congenital agammaglobulinemia (Bruton, 1952). Bruton's agammaglobulinemia is a hereditary, sex-linked, recessive condition affecting only male children with an incidence of about one per 100,000 live male births. Since that time, a large number of primary and secondary immunoglobulin deficiency syndromes have been reported. They include the primary non sex-linked agammaglobulinemia, which is clinically almost indistinguishable from Bruton's agammaglobulinemia, but which has a far worse prognosis; the dysgammaglobulinemias, which are characterized by defective synthesis of one or two of the major immunoglobulin classes; agammaglobulinemias with apparently normal B lymphocytes; common variable immune deficiency syndromes; secondary immunoglobulin deficiencies, which may be either physiological as in the delayed immunological maturation in some infants, or the true acquired hypogammaglobu-

linemias. This latter group may have many etiologies. In addition to the humoral immune deficiency syndrome already mentioned, there are a variety of cellular immune deficiency syndromes that frequently result in hypogammaglobulinemic states. They can include the severe combined immune deficiency syndromes, such conditions as the Wiskott–Aldrich syndrome, and ataxia telangiectasia.

In addition, of course, there are a number of IgG subclass deficiencies that appear to predispose to chronic infections with recurrent exacerbations.

IgG Subclass Deficiencies

The IgG subclass deficiencies constitute a group of primary immune deficiency syndromes that has been investigated in depth only recently. There are four IgG isotypes designated IgG_1, IgG_2, IgG_3, and IgG_4 based upon antigenic differences in their heavy polypeptide chains (Dray, 1960; Terry and Fahey, 1964; Grey and Kunkel, 1964). The relative subclass concentrations in normal serum are 60–70% IgG_1, 14–20% IgG_2, 4–8% IgG_3, and 2–6% IgG_4 (Steinberg et al., 1973). Although each subclass has some activities overlapping those of its the others, other properties are unique to the respective subclass. For example, IgG_1 and IgG_3 fix complement extremely well, while IgG_2 and IgG_4 do so poorly, if at all. IgG_1 crosses the placental barrier easily; IgG_2 hardly crosses at all. Macrophages bind extremely well to the Fc portions of IgG_1 and IgG_3, but not to the Fc of IgG_2 or IgG_4. These characteristics have significance in immune deficiency states. A deficiency of IgG_1 or IgG_3 can result in the inability to fix complement or poor phagocytosis with all the clinical signs and symptoms of an immune deficiency state, yet with significant levels of circulating total IgG. The condition will be revealed only by measurement of subclass levels.

Secondary Immune Deficiencies

Although the value of immune globulin replacement therapy has been confirmed in the primary agammaglobulinemias, absolute evidence of therapeutic usefulness or augmentation with immune globulin concentrates in many of the secondary immune deficiencies is still awaited. Causes of such secondary immune deficiency states include physiological conditions as in the delayed development of the immune system in some infants or as occurs due to insufficient transplacental passage of IgG in premature births; metabolic or other toxemias suppressing the bone marrow as in massive sepsis or renal failure; irradiation or cytotoxic therapy suppressing the bone marrow when used to treat malignant conditions; actual suppression of the bone marrow itself by malignant growths, e.g., myeloma, leukemia of the bone marrow cells; infection and destruction of the cells of the immune system as occurs in acquired immune deficiency syndrome (AIDS); and excess loss of IgG as occurs in burns, protein losing enteropathies, or the nephrotic syndrome. Lack of conclusive evidence of the efficacy of immune globulin therapy in the secondary deficiency states may be attributable to a number of factors. Probably the most important is the inability of today's medical technology to mimic the natural immune response. We can adminis-

ter a polyclonal γ-globulin concentrate prepared from plasma donations from thousands of donors. Yet that concentrate may be lacking a sufficient titer of an antibody directed against the specific antigen constituent of the pathological organism attacking the host. In such cases the organism will overwhelm the passively acquired host immune defense, and if the innate system of the host is unable to supplement the response, then the attacking pathogen will emerge victorious. In the future it may be possible to offset this situation by the selective addition of specific monoclonal antibodies to the polyclonal concentrate. The studies required to prove such benefits either in competition, or better still in combination with antibiotics, will be large and expensive, which equates with a long period before satisfactory proof can be obtained. Other factors are also of importance. For example, in burns Berkman et al. (1988) suggested that in *Pseudomonas* invasion following the burn injury, the elastase produced by the organism is capable of degrading the immune globulin. In addition, in such patients there is rapid immune globulin catabolism and excess loss into extracellular fluids or through the burned surfaces.

Immune and Hyperimmune Globulin Concentrates in Infections

With the resurgence of interest in the immune globulins triggered by the availability of γ-globulin concentrates, resulting from the work of Cohn and his colleagues, and the subsequent description of primary agammaglobulinemia by Bruton, there have been many reports of the beneficial effects of administering pooled human immune globulin concentrates to control the infectious diseases that complicate hypogammaglobulinemias (Gitlin and Janeway, 1956; Domz and Dickson 1957; Janeway and Rosen, 1966; Barandun et al., 1968). Others have investigated the use of immune globulin concentrates in prevention or attenuation of infection, even in immune competent individuals. They include usage in the prevention and attenuation of measles (Ordman et al., 1944), in infectious hepatitis (Gellis et al., 1945; Stokes and Neefe, 1945; Ward et al., 1958; Krugman et al., 1960), and in hepatitis B or serum hepatitis as it was previously known (Mirick et al., 1965; Krugman et al., 1971; Prince et al., 1971; Szmuness et al., 1974; Surgenor et al., 1975). In fact, the latter reports all involved the use of a hyperimmune globulin directed against hepatitis B. By definition, hyperimmune globulins contain at least five times the titer of the specific antibody that would be contained in a normal γ-globulin pool. Although human hyperimmune globulins directed against hepatitis B, against tetanus toxin, and against rabies have been confirmed to be of therapeutic value, others such as those directed against poliomyelitis, mumps, and pertussis have become extinct (Finlayson, 1979). The disappearance of these hyperimmune globulins has in most instances been attributable to the availability of safe and effective vaccines for active immunization (Stiehm, 1988), as well as in certain instances to the absence of proof of efficacy, e.g., in pertussis (Balagtas et al., 1971).

INTRAVENOUS IMMUNE GLOBULINS (IGIV)

In parallel with their increased use, work was being undertaken to modify the immune globulin concentrates, to render them safe for intravenous administration. The advantages of intravenous over intramuscular therapy have been reviewed by Rousell (1981) and Stiehm et al. (1987). Essentially, with the use of the intramuscular route, the dosage is limited by the muscle mass, there is marked pain with increasing volume of injection, there is the possibility of local complications such as abscess formation and absolute contraindications such as bleeding tendencies. In addition an occasional death in infants due to inadvertent intravenous administration of anticomplementary active antibodies has been reported. It also takes 2–7 days to reach maximal blood levels and claims have been advanced that there is local destruction of the immune globulin in the muscle tissue prior to it entering the circulation. The possible loss of bioavailability has, however, never been confirmed in any properly designed experiment. Intravenous therapy ensures 100% bioavailability, the preparation is active immediately, there is minimal pain of injection, and, of course, the only limit to dosage is the risk of cardiovascular overload. An intravenous immune globulin must have none of the limitations of the intramuscular preparation, most important of which is low anticomplement activity. It must not have any loss of activity as a result of modification, there must be adequate intravascular half-life, absence of side effects from impurities, and of course satisfactory physicochemical properties such as stability, reproducibility lot to lot, and proper representation of all IgG subclasses. Many intravenous preparations have been introduced. They range from the highly modified pepsin-digested preparation consisting only of Fab_2, IgG fragments available in Europe since the late 1970s, to the unmodified pH 4.25 preparation first introduced into the United States in the mid-1980s. These preparations are discussed later in this chapter.

Safety of Intravenous Immune Globulins

Most recently there have been concerns about the safety of intravenous immune globulin preparations as regards the possibility of transmitting non-A, non-B hepatitis (NANB). This has been reported by Lever et al. (1984), Ochs et al. (1985), Weiland et al. (1986), Lockner et al. (1987), and Björkander et al. (1988). However, not all intravenous immune globulin concentrates appear to carry the same risk of transmitting non-A, non-B hepatitis (Rousell, 1988; Rousell et al., 1988, 1990). Although the available data are not able to define absolutely why such differences between immune globulin concentrates exist, it does seem reasonable to suspect that the method of fractionation and of finishing the immune globulin concentrate does play an important role in minimizing the possibilities of transmission of non-A, non-B hepatitis.

Current Approved Indications for Immune Globulins

General uses. Today in the United States immune globulin concentrates are approved for usage in immune deficiency syndromes (Tables I, II, and III), to prevent

TABLE I
Uses of Polyclonal Nonhyperimmune Globulins in Humoral Deficiencies[a]

Conditions	Comment
Primary antibody deficiencies	
X Linked agammaglobulinemia	Usual dose 400 mg IgG/kg/month (8 ml/kg 5% IGIV[b] or 2.3 ml/kg 16.5% im ISG[c]); thus monthly dosage best achieved with IGIV
Common variable immune deficiencies	
IgG subclass deficiencies	Studies underway. Caution should be observed in selective absence of IgA, because of possible sensitization to IgA and development of reaginic (IgE) antibodies
Various dysgammaglobulinemias	
Secondary antibody deficiencies	
Physiological hypogammaglobulinemia in infants	IGIV administration may be beneficial until infant's own immune system matures. Studies are underway.
Prematurity/low birth weight infants	To prevent nosocomial infections, studies are underway. Preliminary results in pediatric AIDS are encouraging
AIDS	
Protein losing enteropathies	No clinical results available. Studies should be performed
Nephrotic syndrome	
Antibody deficiencies secondary to malignancies, e.g., multiple myeloma, chronic lymphocytic, leukemia (CLL)	Results encouraging
	Confirmed usage in CLL in dosage of 400 mg/kg every 3 weeks × 1 year. Other studies encouraging
Burns	Results have been disappointing

[a] The intravenous immune globulin may probably be used interchangeably with the intramuscular preparations, provided the difference in IgG concentration is taken into account when calculating dosage.

[b] Intravenous immune globulin—human (IGIV) is usually available as 5% preparation.

[c] Immune serum globulin—human (ISG) given intramuscularly is available as 16.5% solution.

hepatitis A, measles, varicella, and, in certain instances, rubella (Table IV). However, modification of varicella is probably best accomplished by the use of a hyperimmune varicella-zoster immune globulin, while routine use of intramuscular broad spectrum immune globulin for prophylaxis of rubella in early pregnancy is of uncertain value. Some studies do suggest that the use of immune globulin in exposed, susceptible women, can lessen the likelihood of infection and fetal damage, and therefore may be

TABLE II
Uses of Polyclonal Nonhyperimmune Globulins in Combined Immune Deficiencies

Condition	Comments
Severe combined immune deficiencies, including such syndromes as Swiss-type agammaglobulinemia, Gitlin Syndrome, short limbed dwarfism	Definitive therapy requires compatible bone marrow transplantation. Immune globulin therapy may be useful until successful bone marrow grafting

TABLE III
Uses of Polyclonal Nonhyperimmune Globulins in Atypical Immune Deficiencies

Condition	Comments
Ataxia telangiectasia	As there is humoral deficiency with severe decreases in serum and secretory IgA, caution should be observed with usage of IgG. No studies available
Wiskott-Aldrich syndrome	Beneficial results reported with use of IGIV with positive effects on both infections and on thrombocytopenia

considered in individuals who will not consider a therapeutic abortion. In any usage of immune globulins it should always be remembered that the intravenous preparations can be administered in far higher dosage and will provide immediate bioavailability as compared to the intramuscular preparations.

Hyperimmune globulins. The hyperimmune preparations are approved for specific indications (Table V). Hepatitis B immune globulin is indicated for postexposure prophylaxis against hepatitis B. The exposure may be parenteral, such as in needle stick injuries, or may be through mucous membrane contact (accidental splash) or oral ingestion. Hepatitis B immune globulin is also indicated for the prophylaxis of infection in high risk infants born to HBsAg-positive mothers. Rabies hyperimmune globulin is approved for prevention of rabies together with active immunization against rabies in individuals suspected of exposure to rabies, except in those individuals who have been previously immunized with rabies vaccine and have

TABLE IV
Usage of 16.5% Immune Serum Globulins in Infectious Disease[a]

Disease	Objective	Dosage[b] (ml/kg)	Comments
Hepatitis A	Single exposure prophylaxis	0.01	
	Multiple exposure prophylaxis	0.05 every 4–6 months	
Hepatitis B	Postexposure prophylaxis	0.06–0.12	Only if HBIG not available.
Non-A, non-B hepatitis	Prophylaxis	0.12	Not confirmed, but has been used with transfusion.
Rubeola (measles)	Prophylaxis	0.25	Immunocompromised patients get 0.5 ml/kg
Rubella	Prophylaxis	0.55	May prevent fetal damage in pregnancy, but only use of therapeutic abortion refused
Varicella (chicken pox)	Prophylaxis	0.6–1.2	In high risk patients if VZIG not available.

[a] Intravenous immune globulin concentrates (IGIV) contain the same titers of antibody per unit weight of IgG as intramuscular immune serum globulin (ISG) preparations. Thus theoretically it is possible to administer an equivalent dosage of 5% IGIV by multiplying the above doses by 3.3.

[b] Dosage is based upon 16.5% immune serum globulin (human) given by intramuscular injection.

TABLE V
Uses of Hyperimmune Globulins

Disease	Objective	Preparation	Concentration (%)	Dosage[a]	Comments
Hepatitis B	Postexposure prophylaxis	HBIG	16.5	0.06 ml/kg im	
	High risk infants prophylaxis	HBIG	16.5	0.5 ml at birth may be repeated at 3 months	Also hepatitis B vaccine
Tetanus	Postexposure prophylaxis	Tetanus-ISG	16.5	250 units	Also tetanus toxoid
	Therapy	Tetanus ISG	16.5	Up to 6000 units	Also other therapy
Rabies	Postexposure prophylaxis	Rabies ISG	16.5	20 IU/kg	Also vaccine
Varicella-Zoster	Postexposure prophylaxis	VZIG	16.5	12.5 IU/kg	High risk individuals
Rh sensitization	Prophylaxis Rh sensitization in pregnancy	Rh ISG	16.5	300 μg or more	Immediate post-delivery
	Antenatal prophylaxis	Rh ISG	16.5	300 μg	At 28 weeks
	Abortion, amniocentesis, ectopics and other complications of pregnancy	Rh, ISG	16.5	300 μg	At time of event
CMV infection[b]	Prophylaxis in high risk	CMV IGIV	5	Approx. 400 mg/kg/week	Under investigation
Pseudomonas[b]	Prophylaxis in high risk	Ps-IGIV	5	?	Under investigation

[a] This table is intended only as a guide. It is essential to follow carefully the manufacturer's detailed dosage instructions when administering these preparations.

[b] Encouraging results are being obtained in both prophylaxis and treatment of specific infections in susceptible individuals. These products are currently licensed only in Germany.

confirmed adequate rabies antibody titers. Tetanus immune globulin in indicated in patients who require immediate immunity against tetanus toxin, especially those who have little or no preexisting active immunity conferred by past active immunization.

The hyperimmune globulin of especial interest, which is different from those with high titers of antibodies against bacterial toxins or viruses, is, of course, the hyperimmune globulin directed against the Rh_0 (D) blood group. This preparation gives possibly the first hint of the immune modulating capabilities of immune globulin concentrates.

Prior to 1970 Rh hemolytic disease was an invariable result of a pregnancy in which an Rh_0 (D)-negative women conceived an Rh_0 (D)-positive fetus. If the mother did not become immunized against the Rh antigen at the first pregnancy, the risks increased dramatically with subsequent pregnancies, so that the infant at each subsequent pregnancy showed increasing damage as the level of Rh_0 (D) antibodies in the mother increased at each pregnancy. Thus although the infant in earlier pregnancies might suffer from only mild hemolytic disease of the newborn, this would increase with subsequent pregnancies so that the infant from later pregnancies would be born with hydrops fetalis, or erythroblastoses fetalis. Once severe Rh_0 (D) sensitization has occurred, it is impossible for an Rh_0 (D)-negative mother to carry an Rh_0 (D)-positive fetus to term and spontaneous abortion early in pregnancy is frequent. The introduction of Rh_0 (D) immune globulin in 1971 (Pollack et al., 1971a,b) was shown to be capable of reducing Rh isoimmunization in such pregnancies if it was administered to the mother prophylactically within 72 hr of a full-term delivery in all but 1–2% of instances, or even further, reduced to only 0.1% by administering the immune globulin antenatally at 28 weeks gestation, as well as at delivery.

Rh_0 (D) immune globulin is approved for the prevention of isoimmunization in the Rh_0 (D)-negative individual exposed to Rh_0 (D)-positive blood, either as a result of a fetal–maternal hemorrhage occurring during delivery of an Rh_0 (D)-positive infant, abortion (either spontaneous or induced) or following amniocentesis, or abdominal trauma. Similarly, immunization resulting in the production of anti-Rh_0 (D) following transfusion of Rh-positive red cells to an Rh_0 (D) recipient may be prevented by administering Rh_0 (D) immune globulins.

Approved clinical indications for the intravenous immune globulins cover usage for the maintenance therapy of patients who are unable to produce sufficient quantities of IgG antibodies, for example, patients suffering from primary humoral immune deficiency syndrome. In addition, successful usage has been described in idiopathic (autoimmune) thrombocytopenic purpura. In both children and adults, administration of about 2 g of intravenous immune globulin per kg body weight over a period of 2–5 days has initiated a clinically significant increase in platelet count, up to and including complete and permanent remission of the condition.

Other indications for intravenous immune globulins that, although not yet approved in the United States, show remarkable promise as evidenced by the world literature, include usage to control cytomegalovirus infections in bone marrow trans-

plantation, a reduction in graft-vs.-host disease in bone marrow transplantation, prevention of infection in a variety of secondary immune deficiency conditions, as well as usage for immune modulating activity in a number of autoimmune conditions ranging from autoimmune leukopenia, myasthenia gravis, and recurrent abortion. Much work still needs to be performed before the value in these latter conditions can be confirmed.

CHEMISTRY OF IMMUNE GLOBULIN G

Structure of IgG

A great deal is known about the structure of human IgG molecules, and the reader is referred to an excellent overview by Stryer (1981) and a detailed review by Poljak (1978). Specific aspects of this structure work will be covered, as well as more recent work on molecular dynamics of IgG that are important to understanding the effects that preparative techniques have on the functional properties of antibodies (McCue, 1989).

Antibody (of the IgG class) to any particular antigen is a heterogeneous group of molecules differing in antigen or complement-binding affinity. Structurally the antibodies may differ by as little as a single amino acid substitution at the antigen-binding site, or as great as multiple amino acid substitutions elsewhere in the molecule. Structurally all IgG molecules appear by electron microscopy to be predominately "globular" in nature with "Y"- or "T"-like topology. The molecules display molecular flexibility about the intersection of the three arms of the "Y," and therefore are said to have a "hinge region." On close examination by X-ray diffraction analysis, each arm is found to consist of two polypeptide chains that are held in alignment with each other, predominately by noncovalent bonding. Two of the chains run the length of the molecule (H-chains); the other two are about half their length (L-chains) and are covalently anchored to the H-chains by one interchain disulfide bond stemming from cysteine at the C-terminus of each L-chain. Each chain in each arm of the molecule has two intrachain disulfide bonds that assist in folding the chains into two hydrophobic domains responsible for the globular nature (a total of eight globular domains) of the molecule in solution. The hinge region of the molecule is a short interval of amino acids adjacent to a series of disulfide bonds between the two H-chains, about midpoint along the length of the molecules.

IgG Subclasses

Some structural features among the diversity of antibodies are so well controlled at the gene expression level that readily recognizable subclasses of IgG are identifiable. Most notable are the number and location of disulfide bonds. The molecular structures of the human IgG_1 and IgG_4 subclasses have two interchain disulfide bonds adjacent to their hinge regions, but differ as to where the L-chains are covalently attached to the H-chains. In IgG_1-type molecules the L-chains are covalently linked to

the H-chains about half way down the molecule and adjacent to the hinge region. In IgG_4-type molecules the L-chains are covalently linked to the H-chains about one quarter way down the molecule from the N-terminus, between globular domains, and thus distant from the hinge region. The molecular structure of the IgG_2 subclass has four interchain disulfide bonds between the H-chains in the hinge region and attachment of the L-chains is similar to that found in IgG_4 type molecules. From a topological viewpoint IgG_3 is quite different from the other subclasses with an elongated region of interchain disulfide bonds (i.e., 11 adjacent to the hinge region), while attachment of L-chains to H-chains is similar to that in IgG_2 and IgG_4. In all subclasses the interchain disulfide bonds associated with the hinge region are at the surface of the molecules and therefore exposed to bulk solvent. The intrachain disulfide bonds associated with the globular domains of the molecules on the other hand are relatively buried within the molecule.

Proteolytic Cleavage of IgG

The various topologically distinct features of IgG can be cleaved from the intact molecules by action of the proteolytic enzyme papain. If the cleavage is carried out under mild conditions, the three arms of the molecule are obtained as separate molecular entities. Two of these are identical in amino acid composition, but differ slightly in geometry. Both are soluble fragments that bind antigen, and are therefore called the Fab (fragment-antibody) arms of the intact molecule. The third fragment precipitates from solution and was therefore named the Fc (fragment-crystalline) arm of the intact molecule. This material binds the C1q component of the complement system. Further attack on the Fab components by proteolytic enzymes such as pepsin separates the globular domains of the arms. These globular domains are of two types, those with highly variable amino acid sequence (i.e., variable domains V_L and V_H) and those of highly conserved amino acid sequence (constant domains C_L and C_H1). X-Ray diffraction analysis shows that this cleavage occurs at specific stretches of the polypeptide chain exposed to bulk solvent. These stretches, which link the globular domains of variable amino acid composition to the globular domains of constant amino acid composition are the "switching" regions of the Fab arms. The variable domains of the L-chain and H-chain interact through noncovalent bonding to form, with the remaining open tail at the N-terminus of the chains, a pocket that is the antigen-binding site of antibodies. Stronger proteolytic cleavage of the Fc arms yields the topologically distinct globular domains of this fragment. The globular domain adjacent to the hinge region (i.e., C_H2) has been shown to contain the binding site for C1q of the complement system (Endo and Arata, 1985), while the C_H3 globular domain is believed to contain the binding site for cell surface attachment (Dorrington and Klein, 1983), including binding to protein-A of staphylococcus and protein-G of streptococcus bacteria. Taken together, the various globular domains of constant amino acid composition make up two-thirds of intact antibodies. Therefore, it is assumed that these are responsible for the structural and functional integrity of the antibodies.

IgG also contains carbohydrates (2.9% by weight for human), most of which are covalently linked to the polypeptide chains of the Fc portion of the molecules. As such, the carbohydrates separate the two C_H2 domains and allow them to function independently. The exact physiologic role of these carbohydrates is, however, not known. Variation in the constituent monosaccharides and the presence of amino sugars and sialic acids suggest a role involving cell receptors.

Flexibility of IgG

Since the earliest attempts at visualizing the topology of IgG molecules (Feinstein and Rowe, 1965; Noelken et al., 1965), it has been recognized that these molecules display segmental flexibility (Cathou et al., 1976). Various studies using fluorescence depolarization techniques have indicated that the Fab portions of IgG not only display a variable angle between them, which accounts for the "T" and "Y" static configurations seen by electron microscopy and X-ray diffraction techniques, but also rotation of each Fab domain about their point of attachment at the hinge region of the molecule. The time frame of the rotations detected by fluorescence was on the order of 10 to 100 nsec. More detailed studies of the hinge region of the IgG indicated that a short stretch of the H-chain involving the interchain disulfide bonds is the point at which segmental flexibility occurs. From this it is assumed that at least one functional purpose for the interchain disulfide bonds is to anchor the hinge and stabilize the backbone structure of the molecule during the rotational and angular movement of the Fab domains. This assumption has been investigated by comparing "native" antibodies with those in which interchain disulfide bonds were broken. Chan and Cathou (1977) found that reduction and alkylation of the interchain disulfide bonds in the hinge region of IgG resulted in greater angular movement of the Fab arms relative to each other, and this has been associated with loss in functional integrity of IgG (Law and Painter, 1986).

Movement of entire Fab arms about the hinge region of IgG is not the only pronounced flexibility displayed by these molecules. Kaivarainen and Nezlin (1976) observed two conformations within Fab segments of IgG in which the distances between variable (V_L and V_H) and constant (C_L and C_H1) globular domains are different. The lifetime of these conformations appeared to be greater than 100 nsec. One conformation (i.e., the "looser" one) was assumed to be preferred by IgG in solution in the absence of antigen, and the other conformation (i.e., the "tighter" one) was preferred by IgG when bound to antigen.

Conformational Changes in IgG

Because binding of antigen by antibodies causes functional changes in the antibodies at points distant from the antigen-binding site, it has been assumed that conformational changes throughout the molecule are responsible for these functional changes. However, efforts using CD spectroscopy (Steiner and Lowey, 1966; Cathou et al., 1968) and X-ray diffraction analysis (Amzel et al., 1974; Segal et al., 1974) to see such

changes have failed to find them. Cathou et al. (1968) concluded that the conformational change, therefore, must be small and manifested primarily in the quaternary or tertiary structure and not in the secondary structure of the molecules. Recently, however, it has been reported (McCue et al., 1988) that conformational changes in the secondary structure of IgG in solution were dependent on how the IgG was purified. Figure 1 shows repetitive scans of the CD spectrum of polyclonal human IgG isolated and formulated at pH 4. At this pH an absorption band of oscillating optical orientation is seen near 200 nm; while at physiologic pH the band is not seen above background. The position of this band corresponds well with the absorption near 200 nm for peptide bonds in the UV spectrum of IgG (Figure 2). Because the band near 200 nm is absent from the CD spectra of IgG at physiologic pH, the CD band near 220 nm was assigned in earlier work to the peptide backbone of these molecules. In Figure 2, however, it can be seen that the CD band near 220 nm arises from absorption of tryptophan side chains at 220 nm. Various other data on the physical and functional differences between IgG isolated and formulated at various pH levels indicate that long-term exposure of purified IgG to physiologic buffers results in denaturation of the molecules. This denaturation may be responsible for the absence of detectable conformational changes between free and antigen-bound antibody reported earlier.

Functionally Significant Molecular Motions

The range of functionally significant molecular motions may not be limited to movement about the hinge region or the switching regions of IgG molecules. In general, a wide variety of molecular motion can be seen with globular proteins (McCammon and Karplus, 1980). These motions occur on time scales ranging from seconds to hours. Of particular interest are low-frequency vibrations of the molecule that suggest motion on a large or collective scale within the molecule (Karplus and McCammon, 1981). From a topological point of view these low-frequency motions of globular proteins are seen as "breathing" motions of the molecules (Pickover, 1984). For antibodies, this breathing motion may be as important as structural fit for binding of antibody to antigen (Marx, 1984; McCue et al., 1988).

Precipitation From Aqueous Solutions

The globular nature, size, and amphoteric properties of immune globulins make it relatively easy to precipitate these molecules from aqueous solutions. Addition of sodium or ammonium sulfate to serum, plasma, ascites fluid, or tissue culture media will "salt out" the immune globulins by promoting aggregation. The basis of this is believed to be the movement of solvent water from the hydration sphere of the protein to that of the salt (Cohn and Edsall, 1943; Czok and Buecher, 1960; Dixon and Webb, 1961), thus lowering the degree of protein solvation. However, this model ignores the ability of some anions to participate in intermolecular bridging. Even chloride ion is known for its ability to form bridges between complex and steric hindered molecules (McCue, 1973) and promote aggregation of purified IgG (McCue et al., 1986). Cations capable of forming bridged complexes will also promote precipi-

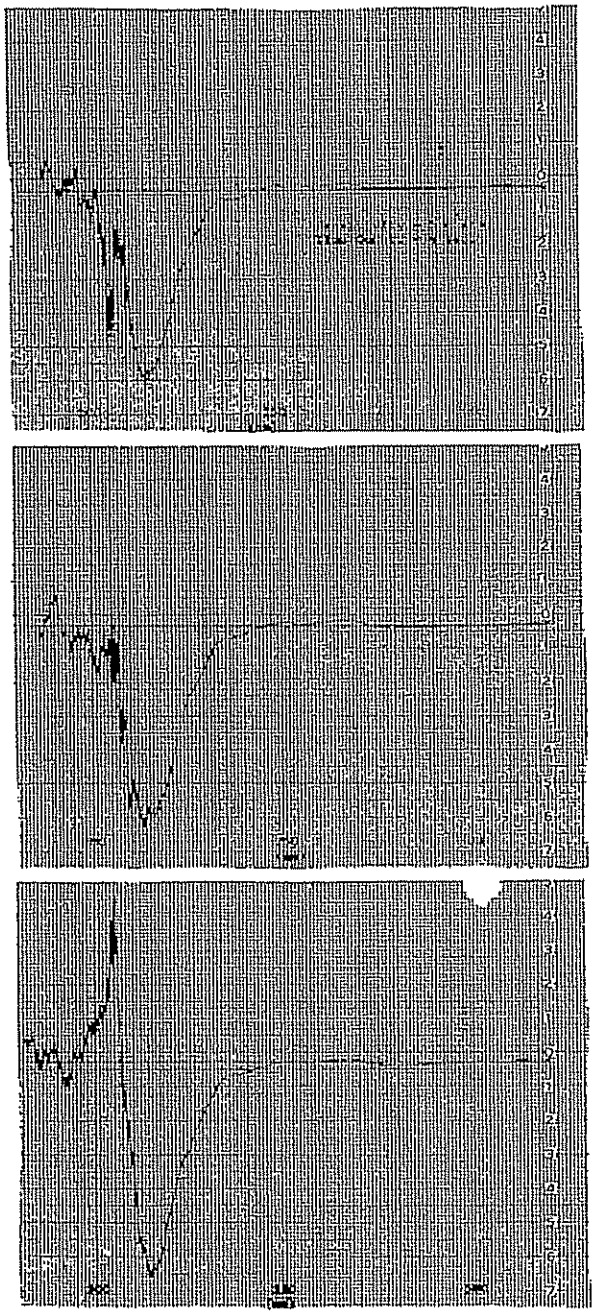

Fig. 1. Circular dichroism spectra of polyclonal human IgG in water at pH 4. Each spectrum is a single scan of the IgG solution run on a Jasco-500C spectrometer from 320 to 190 nm under

tation of immune globulins (Cohn et al., 1950). In both the anion and cation promoted precipitation of immune globulins the rate and extent of precipitation are at their maximum in solutions at the isoelectric point of the proteins. Under such conditions protein–protein charge repulsion is at a minimum, thus allowing closer approach of the molecules. The forcing of immune globulins out of aqueous solutions by restricting the availability of solvent water to the proteins does appear to take place when water-soluble organic molecules such as ethanol and polyethylene glycol (PEG) are added to aqueous solutions of these proteins (Polson and Ruiz-Bravo, 1972).

IMMUNE GLOBULIN G FRACTIONATION

Cold-Ethanol Fractionation for Commercial Scale Manufacturing

The common technique used to isolate γ-globulins from human plasma for clinical use is precipitation from cold-ethanol/water mixtures according to Cohn et al. (1946). Cohn Method 6 has become the starting point for isolating safe and efficacious IgG.

The Choice of an IgG Concentrate

The choice of an IgG concentrate for therapeutic purposes was probably based upon two major factors:

1. IgG is the immune globulin present in greatest concentration in normal serum and hence will give the best yield in any plasma fractionation process.
2. The most frequently diagnosed and reported immune deficiency state was Bruton's X-linked hypo- or agammaglobulinemia.

Since these early influences, other factors confirm the therapeutic choice of IgG:

1. IgG is distributed through both the interstitial and intravascular spaces, while IgM is confined to the intravascular space. The active dimer of IgA (secretory IgA) exerts its biological activity only in mucous membrane secretions, while IgE becomes fixed to mast cells and basophils to exert its reaginic activity. IgG is, of course, also the only immune globulin to cross the placental barrier.
2. IgG is the immune globulin primarily involved in the secondary immune response. As it provides immune protection in both interstitial and intravascular spaces, it is capable of assuming the protective function of IgM. The converse is not true.
3. Because it is distributed through both interstitial and intravascular spaces, IgG can be administered either intravenously or intramuscularly. IgM concentrates must be given intravenously.

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nitrogen using a far UV. The scan rate was 5 nm/min, the sensitivity setting was 5 m°/cm, and the time constant was 4 sec.

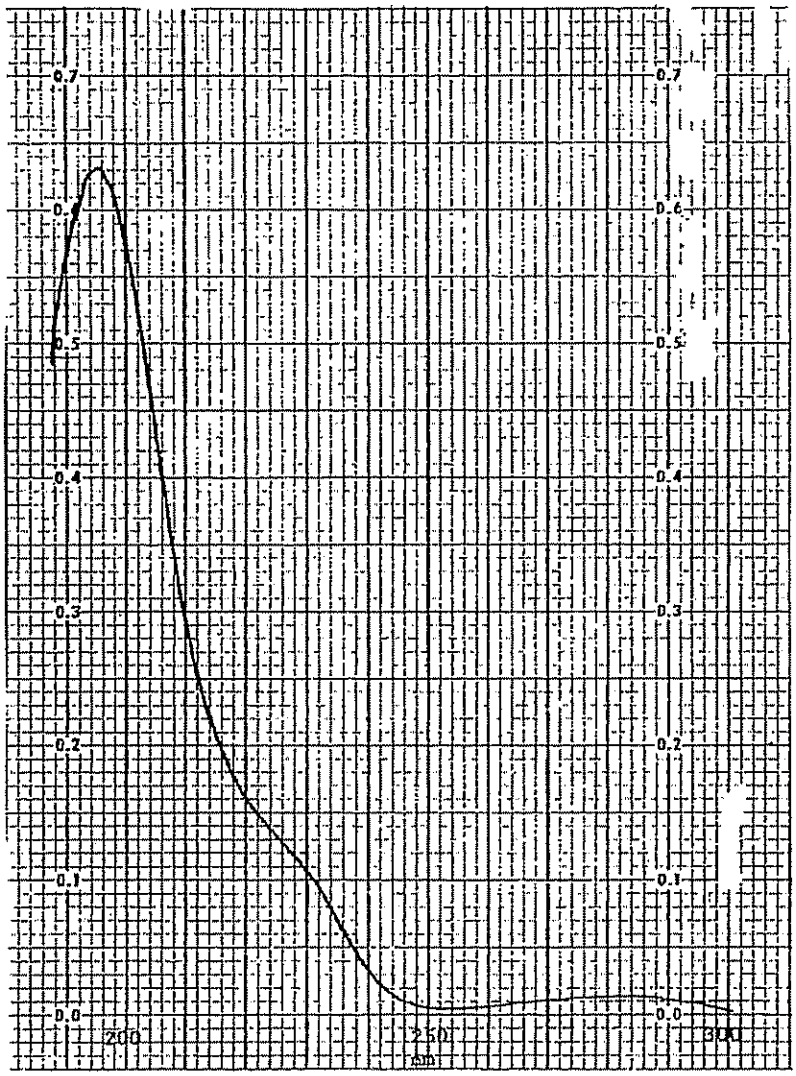


Fig. 2. Ultraviolet spectrum of polyclonal human IgG in water at pH 4. The spectrum was run on a Cary-14 spectrometer from 300 to 185 nm under nitrogen using a far-UV quartz cell.

4. The blood group isoagglutinins (anti-A and anti-B) are of the IgM class and thus hemolytic reactions are less likely with IgG concentrates.

5. Partitioning of viruses during the fractionation procedure is less likely with the IgG than with the IgM fractions.

6. IgM exists as a pentamer and is thus capable of spontaneously activating complement, while IgG will fix complement (in the absence of specific antigen) only if

aggregation occurs. Thus IgG concentrates are less likely to induce complement-mediated side effects when given intravenously.

Two Cold-Ethanol Fractionation Procedures

Two specific approaches for fractionation of IgG from plasma are of particular interest, that of Oncley et al. (1949) and that of Kistler and Nitschmann (1962). These are the methods used by commercial suppliers of clinical grade IgG. (See Nezlin, 1977 for a discussion of cold-alcohol fractionation of plasma used in the Soviet Union.) Figure 3 shows the important parameters of the Oncley, and Kistler and Nitschmann methods. The principal difference between the two is that Oncley Method-9 resuspends and re-precipitates the gamma globulin fraction from Cohn Method-6 before removing the IgM by precipitation, while the Kistler and Nitschmann method does not.

Even though the two methods appear to be virtually the same, controversy has arisen over whether or not the IgG products from the Oncley method and that from the Kistler and Nitschmann method are identical. When the controversy has turned to questions of transmitting hepatitis, strong opinions have been expressed (Friedi and Morgenthaler, 1985). Although infectious episodes have occurred with IgG obtained from these purification methods, they are not due simply to fractionation of contaminated plasma. Work by Murray and Ratner (1953) demonstrated that IgG isolated from infectious plasma by the Cohn/Oncley method does not transmit hepatitis. The reason for this was demonstrated by Pennell (1957) as the ability of the Cohn/Oncley method to physically remove viruses from the IgG fraction; an observation confirmed for hepatitis B virus by Schroeder and Mozen (1970) and Hoofnagle et al. (1976) and for human immunodeficiency virus by Wells et al. (1986). Similar results were reported by Leveque et al. (1975) and by Henin et al. (1988) for IgG purified by the Kistler and Nitschmann method. The occasional episodes of hepatitis infection arising from specific lots of IgG purified by these cold-ethanol fractionation methods are the result of not conducting the fractionation steps in a manner that maximizes removal of viruses when precipitating IgM from the Cohn γ-globulin fraction. In addition, if the IgM precipitation is not done in a manner that maximizes coprecipitation of virus, it will also not maximize removal of unwanted plasma proteins. The amount of virus and unwanted plasma proteins precipitated with IgM increases with increasing EtOH concentration, decreasing temperature, and increasing pH. Our experience has been that at 17% EtOH and $-6°C$ the purity of IgG obtained is optimal when the precipitation of IgM is carried out at pH 5.35 (measured on samples diluted 1:5 in 0.15 M saline) while at lower pH traces of other proteins (Figure 4) and viruses can be found, and at higher pH the IgG yield decreases significantly. The Kistler and Nitschmann method takes these issues into account by precipitating IgM at 12% EtOH and pH 5.1 to minimize the loss of IgG, and then cleans up the IgG by precipitating it from contaminating proteins in 25% EtOH at pH 7.0. However, the protein purity of the IgG from the Kistler and Nitschmann method is not equivalent to that obtained from

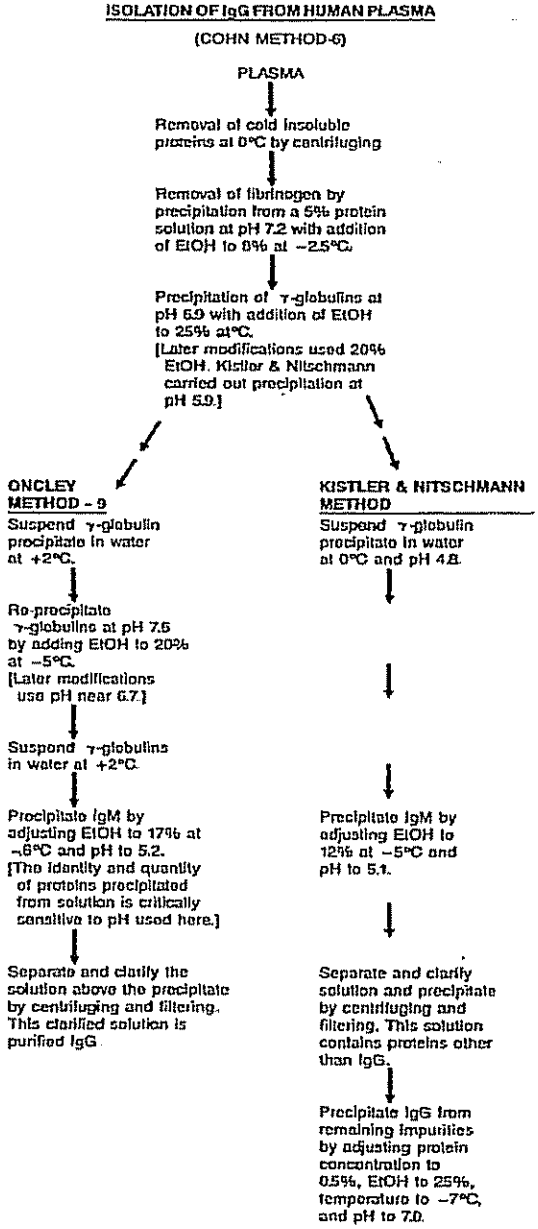


Fig. 3. Comparison of process steps for the Oncley, and the Kistler/Nitschmann plasma fractionation methods.

the Oncley method before or after a similar precipitation step following removal of IgM (McCue and Johnson, 1986).

In addition to the conditions for precipitating IgM, the method of removing the precipitate from solution can significantly affect the quality of the final IgG product. For commercial scale fractionation, the bulk of IgM precipitate is removed by continuous flow centrifugation followed by depth filtration and finally membrane filtration. To achieve optimum purity of the final IgG solution, the filters should not be rinsed after filtration (a practice sometimes used to improve IgG recovery) since many unwanted proteins are only coprecipitated with IgM and will wash out of the precipitate.

Other Fractionation Procedures

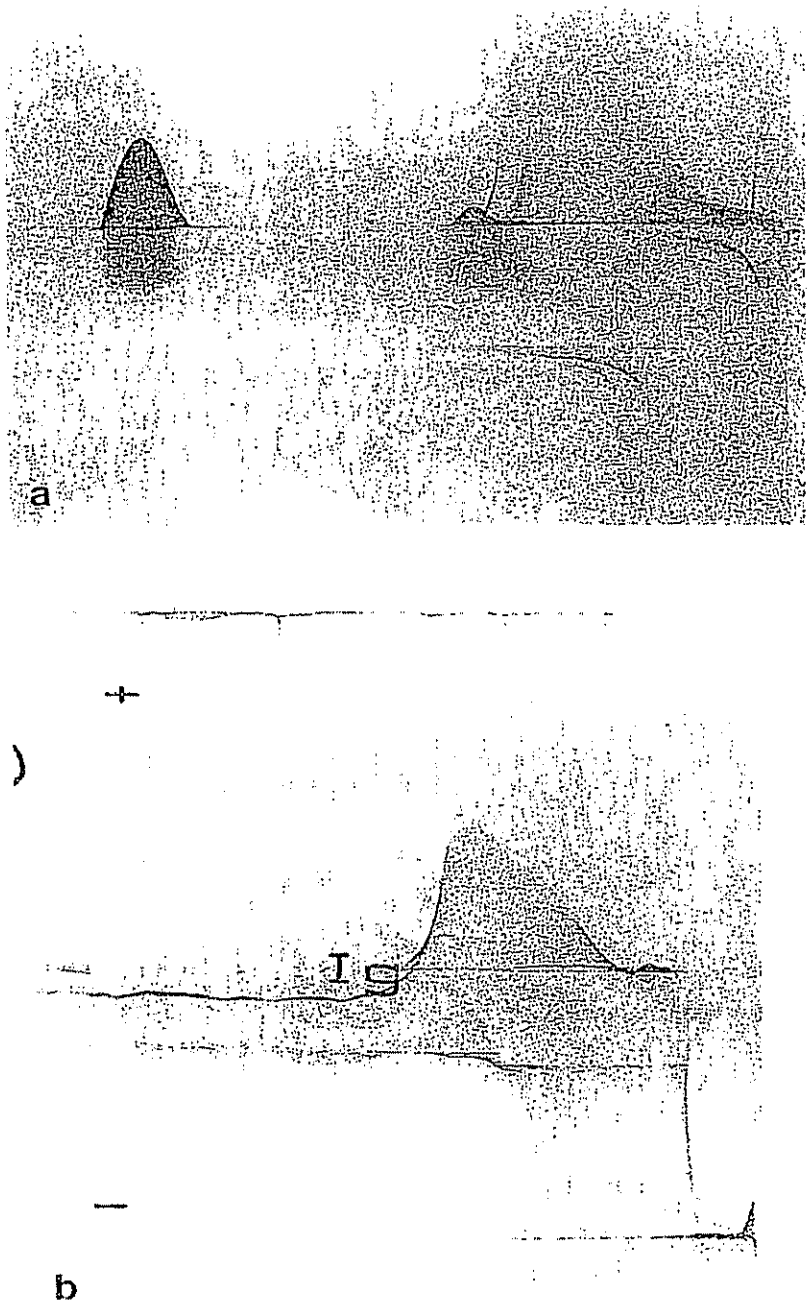
In addition to cold EtOH fractionation of plasma for purifying IgG, other methods such as those involving direct isolation of IgG from plasma by ion-exchange chromatography (Condie, 1981), or polyethylene glycol precipitation (Polson and Ruiz-Bravo, 1972) have been used, but concerns about transmitting hepatitis have eliminated these as primary purification methods for licensed commercial products.

Monoclonal Antibodies

Even though all commercially available antibodies licensed for clinical use are isolated by cold-EtOH fractionation of human plasma, monoclonal antibodies produced in tissue culture from hybridoma cells or from Epstein–Barr virus-transformed human cells are becoming available for therapeutic use. The cost of antibody source materials for a monoclonal can be 5 to 50 times that of human plasma per milligram of specific antibody, therefore, purification processes must be more efficient than the cold-EtOH fractionation which recovers about 50% of the IgG found in the source material. Because of this, there is renewed interest in using the more efficient IgG isolation methods abandoned for use with plasma.

The first step in recovering monoclonal antibodies from tissue culture is separation of the cell mass from the culture media into which the antibodies have been secreted. If the volume of supernatant is >100 liters, continuous flow centrifugation is the most suitable method for accomplishing separation. For supernatant volumes < 2 liters, filtration is possible but troublesome. The next step is concentration of the culture media by ultrafiltration to make the processing volume more manageable. At this point low-molecular-weight materials can be removed and ionic strength adjusted as needed by diafiltration. After diafiltration the IgG can be separated from other proteins by ion-exchange chromatography (Levy and Sober, 1960), ammonium sulfate precipitation (Cohn et al., 1940), or affinity chromatography.

Single step ion-exchange chromatography (Levy and Sober, 1960) has the advantages of being gentle, rapid, with a high volume throughout, and inexpensive. But recovery of IgG is about 70%, varies with the physical properties of the specific antibody being isolated, and the IgG is contaminated with trace amounts of proteo-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lytic enzymes, polynucleotides, and viruses. The contaminating materials can be separated from the antibodies by further ion-exchange chromatography or by size-exclusion chromatography, however, additional loss of IgG will occur.

Isolation of monoclonal antibodies by fractional precipitation at pH 6 with 1.39 M ammonium sulfate (Cohn et al., 1940) gives a less pure preparation than that obtained by single-step ion-exchange chromatography, but is capable of isolating antibodies of the IgM class as well as IgG. After precipitation the antibody is dissolved in water and diafiltered to remove the ammonium sulfate. The antibody must then be further purified from contaminating proteins, polynucleotides, and viruses.

An idealized strategy for purification of monoclonal antibodies is to recover 100% of the available material by affinity chromatography. The feasibility of this depends on the availability, stability, cost, and clinical significance of the receptor proteins to be used as the immobilized affinity agent. In most cases where antigens are used, one or more of these factors is not favorable. An alternative approach to the use of specific antigens is the use of protein-A from the cell wall of staphylococcus bacteria or protein-G from specific strains of streptococcus bacteria (McGuire, 1989). In general protein-G appears to have advantages over protein-A for isolating monoclonal antibodies in that it binds human IgG subclass 3 better than protein-A does. One disadvantage with native protein-G is that it also binds albumin and thus makes it difficult to remove traces of proteins and polynucleotides that normally bind to albumin. This problem, however, has been eliminated by using recombinent (r)-DNA techniques to engineer modified protein-G in which the albumin binding domain is absent (GeneX Corp., Gaithersburg, MD, USA). With this material covalently coupled to a solid support such as agarose or silica beads, greater than 90% of available IgG can be recovered from antibody source material in high purity with a simple procedure. IgG source material is diluted in phosphate buffer near pH 7 and loaded onto the immobilized protein-G. Unwanted proteins, polynucleotides, and viruses are washed away with elution buffers varying from pH 7 to 5. The IgG is then unloaded from the protein-G using acetate buffers between pH 3.0 and 3.5. In our hands r-DNA protein-G immobilized on silica is very stable and has been able to withstand the repeated use and abuse of students learning to purify antibodies.

A number of companies using protein-A and protein-G for purifying monoclonal antibodies for therapeutic use have product license applications before the U.S. Food and Drug Administration, and a critical question yet to be resolved with these applications is what is a safe level of protein-A or protein-G leaching into the IgG solution

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Fig. 4. Crossed immunoelectrophoresis patterns of human IgG prepared from Cohn Fraction II + III after precipitating IgM at (a) pH 5.2 and at (b) pH 5.4. Cathodic and anodic moving IgG appears at the far right in both (a) and (b). Contaminating plasma proteins appear in (a) to the left of the IgG bands. The IgG in (a) was also found to have 17% fragmentation due to contamination with proteolytic enzymes.

during purification. With presently available immobilized protein-A and protein-G, 3–8 ng of these proteins is found per milliliter of antibody solution. Although this appears to be significant it is important to note that plasmapheresis on immobilized protein-A has been licensed and in use for several years. Since plasmapheresis involves total plasma volume contact (~3 liters) with the immobilized protein-A, and administration of monoclonal antibody for therapeutic use involves milliliter doses, the presence of nanogram per milliliter quantities of protein-A or protein-G in monoclonal antibodies is not expected to result in clinical problems. In addition, the control of pyrogen accumulation on these affinity columns must be addressed. Fortunately, r-DNA modified protein-G is very stable and can withstand pH treatments between 2 and 10. This should permit the use of accepted methods for controlling pyrogens on hollow fiber ultrafilters, which is to clean with dilute phosphoric acid followed by dilute NaOH solution.

Process Control

The quality and lot-to-lot reproducibility of purified antibodies are significantly influenced by process control as well as process design. For reasons discussed earlier, the ability to control pH to within 0.05 units is necessary in order to precisely control the balance between purity and yield of the final product. In the cold-ethanol fractionation methods, the pH should be determined using pH electrodes in solutions of low alcohol concentration. This is accomplished by diluting a test sample from the batch process 1:5 with distilled water or 0.15 M saline at room temperature before making the pH measurement. It is important to note that dilution made in saline, or other diluents, will give different pH readings from those observed in water.

Such an approach to the control of pH is cumbersome and prone to sample variability when carrying out large-scale fractionation runs. From experience, the greatest source of error with pH measurement has been the maintenance of the electrodes. Fouling of the pH-sensitive glass electrode surface can occur if the electrode is allowed to dry out while in contact with protein. This changes the slope of Nernstian response of the electrode. Even with careful attention to cleaning, the porous junction of the reference electrode will eventually become fouled on repeated or prolonged contact with protein solutions. Such contact results in a change of junction potential for the electrode.

In short, protein fouling of the electrodes gives rise to slope and intercept changes in electrical response to hydrogen ion concentration. Although these changes in calibration can be compensated by frequent recalibration, accompanying changes in response time required for the electrodes to come to equilibrium with the solution will eventually lead to problems that cannot be remedied by recalibration. For example, extensive protein precipitation in the porous junction of the reference electrode will result in unstable pH readings. This can be remedied for a limited time by soaking the electrodes after use in dilute aqueous HCl, but it is far better to discard the electrodes when their response time becomes greater than 1 min.

Various attempts have been made to use pH sensing electrodes on-line in fractionation processes. The most successful attempts have been with reference electrodes

constructed to accommodate a slight positive pressure inside the electrode. This pressure reduces the tendency for protein to enter the porous junction of the electrode where it will precipitate. Improvement in the performance of the pH sensing glass electrode can be achieved by rapid movement of protein solution to produce shear forces great enough to keep the electrode surface from fouling. Movement of solution also reduces electrode response rate by no longer making it diffusion controlled. Under such conditions, pH measurements reliable to ±0.05 units were obtained for 20-hr continuous contact with plasma proteins (McCue, 1974), after which the electrodes required cleaning and recalibration.

There is, of course, no equivalency between pH measured in-process and pH measured in diluted samples. Alcohol concentration, ionic strength, and temperature differences in-process affect pH values. Most important among these is the alcohol concentration. The pH of phosphate-buffered solutions (pH 7.0) and acetate-buffered solutions (pH 4.0) increases linearly with increasing ethanol up to 40% alcohol by volume. The change is 0.016 pH units for each percent ethanol added to the phosphate buffer, and 0.023 pH units for the acetate buffer. This relationship between ethanol concentration and pH is unaffected by buffer concentration. Varying the temperature from 0 to 25°C moves the pH up and down according to the temperature dependency normally seen with the buffers in the absence of alcohol.

Measurement and control of solution ionic strength are also critical to composition and yield of final product. In the Oncley method, all that stands between precipitation of IgM as Fraction III and precipitation of an IgG subfraction as Fraction II-3 is a change in NaCl concentration from 0.015 to 0.05 M. The remainder of the IgG is obtained by precipitating Fraction II-1,2 from the solution after adjusting pH from 5.2 to 7.4 and ethanol from 17 to 25%. These subfractions differ in antibody composition (Janeway et al., 1946) and IgG subclass (Table VI) distribution. The most commonly used technique for determining ionic strength is calculation of the salt concentration in solution. With the advent of rapid, large-scale diafiltration systems very precise adjustment of ionic composition can now be achieved, if ionic strength is monitored directly in solution by conductance measurement; the use of a highly polished gold electrode in contact with the solutions is preferable because these are not as easily fouled as other types of electrodes.

Variation in chemical composition of solutions caused by contact with process equipment must also be considered in process control. For example, ppm amounts of metal ions, such as copper (Gerber, 1974), can influence IgG aggregation and thermal stability. Analysis of various lots of commercial IgG products by inductively coupled plasma (ICP) emission spectroscopy has found variable amounts of Fe, Cu, Zn, Ni, Co and Al at the 0.020–0.200 ppm level in products (McCue, 1984).

FINISHING OF IMMUNE GLOBULIN FOR INTRAVENOUS USE

Important differences among IGIV (immune globulin intravenous) products today are the methods used to control anticomplement activity of the purified IgG and the

TABLE VI
Composition of IgG Subfractions

IgG subfraction	IgG (%)	IgM (%)	IgA (%)	IgG_1 (%)	IgG_2 (%)	IgG_3 (%)	IgG_4 (%)
Frll-3	98.54	0.6	0.86	58.2	29	6.6	6.2
Frll-1,2	98.55	0.56	0.88	61.4	32.3	3.7	2.6

TABLE VII
Some Commerical IGIV Products

Manufacturer	Product	A/C Control	Formulation
Cutter	GamImune-N	Ultrafilter Diafilter at pH 4	5% igG in water with 10% maltose at pH 4.25
Sandoz	Sandoglobulin	Mild pepsin digestion at pH 4	5% IgG freeze dried with 5% sucrose at pH 6.8. Diluent: 0.9% NaCl [trace]
Hyland-Travenol	Gammagard	PEG treatment, and albumin	5% igG freeze dried with 0.9% NaCl, 0.3 M glycine, 2% glucose, 0.1% albumin, 0.2% PEG at pH 7.0. Diluent: water
Biotest	Intraglobin F	β-Propiolactone treatment at 37°C	5% IgG in water with 0.45% NaCl, 2.5% glucose at pH 6.8
Immuno	Endobulin	Peptidase digestion at pH 4 and PEG treatment	5% IgG freeze dried with 0.3% NaCl, 5% glucose, 0.5% PEG at pH 7.0. Diluent: water
Green Cross	Veno-globulin-I	PEG treatment	5% IgG freeze dried with 1% albumin, 0.5% PEG, 2% mannitol, 0.5% NaCl at pH 6.8. Diluent: water
Kabi-Vitrum	Gammonativ	Formulation with albumin	5% IgG in water with 0.4% glycine, 0.08% caprylate, 5% albumin, 2.4% glucose, 0.1% acetyltryptophan at pH 6.8
Teijin Institute	Venilon	Sulfonation	5% IgG freeze dried with 2.25% glycine, 0.25% albumin, 1% mannitol, 0.9% NaCl at pH 6.8. Diluent: water
Behringwerke	Venimmun	Sulfonation	5% IgG freeze dried with 0.3 M glycine, 0.9% NaCl. Diluent: water

final formulation of the products. TABLE VII summarizes these differences for several commercially available products.

Control of IgG Anticomplement Activity

Once IgG has been isolated from the other bulk proteins of plasma, it is more susceptible to deterioration. At physiologic pH purified IgG readily self-associates in solution and becomes thermally unstable (McCue et al., 1986), and on standing forms aggregates that have been shown to spontaneously activate the complement system in vivo (Barandun, et al., 1962). These changes are associated with adverse clinical reactions that often are life-threatening if the IgG is given intravenously and, therefore, led early to restricting IgG to intramuscular use (Oncley et al., 1945). Starting from the pioneering work of Barandun et al. (1962) various approaches to preventing or removing the anticomplement activity of cold-EtOH-purified IgG have emerged and found their way into commercial products (Romer et al., 1982a; Lundblad et al., 1986; Lundblad and Londeree, 1988). These approaches fall into three groups: removal of IgG aggregates, modification of IgG molecular structure, and stabilization of IgG. Removal of aggregates from Oncley or Kistler and Nitschmann IgG fractions can be done using PEG (Polson and Ruiz-Bravo, 1972), with residual amounts of PEG in the final product appearing to prevent further aggregation (Lundblad and Londeree, 1988) and anticomplement activity (Romer et al., 1982b; Hooper et al., 1984). Modifications of IgG molecular structure that eliminate detectable anticomplement activity are the breaking of disulfide bonds in the molecules using reduction and alkylation (Pappenhagen et al., 1975) or sulfonation (Gronski et al., 1983; Hofstaetter et al., 1983), reaction with β-propiolactone (Stephan, 1975), and digestion with proteolyte enzymes (Barandun et al., 1962). These methods, however, appear to degrade IgG subclass 3 antibodies (Heiner, 1984).

In the development of IGIV products, recent emphasis has been focused on more gentle processing of purified IgG in order to avoid aggregation and anticomplement activity brought on by exposure to EtOH and freeze-drying. Thus the use of diafiltration and ultrafiltration (Vandersande et al., 1982) to remove EtOH and concentrate IgG has become common. When this is done at pH 4, a stable, water clear, monomeric IgG solution is obtained (McCue et al., 1986). This material, when formulated with 10% maltose to produce an isotonic solution, is free of serious adverse clinical reactions (Rousell, 1988). This alternative to precipitation at pH 7.2 for concentrating IgG, and freeze-drying for removal of EtOH yields antibodies with better antigen-binding affinity (McCue et al., 1988). An alternative approach to stabilizing purified IgG at low pH is the addition of human albumin to the IgG solution; the albumin interferes with IgG aggregate formation at physiologic pH and during freeze-drying, and limits development of anticomplement activity (Janeway et al., 1946).

Control of Proteolytic Enzymes

A different type of stability problem results when proteolytic enzyme activity is present in the final IgG product (Art and Finlayson, 1969). Proteolytic enzymes cause

fragmentation of IgG and loss of antibody titers. Whether or not such activity is detectable varies from lot to lot and manufacturer to manufacturer of IgG products. Painter and Minta (1969) showed that one source of this variability is the pH at which IgM is precipitated from the Cohn γ-globulin fraction. Precipitation of IgM at pH less than 5.4 results in significant levels of proteolytic enzyme activity being found in IgG products while precipitation at pH greater than 5.4 leaves at most traces of detectable activity. Other sources of the problem are nonoptimal removal of the IgM precipitate from the IgG solution, and the washing of the IgM precipitate in order to optimize IgG recovery. Elimination of proteolytic enzyme activity can be achieved by anion-exchange chromatography, PEG reprecipitation, and formulation at pH less than 6.4 (Art and Finlayson, 1969).

Formulation

Aside from the use of pH, albumin, and PEG for controlling anticomplement activity, there has been little attention given to the affects of other "excipients" commonly used in formulating IGIV products. As early as 1945 it was recognized by Oncley, Enders, and Janeway that formulation with saline was not advisable because it caused turbidity, aggregation, thermal instability, and loss of antibody titers (Oncley et al., 1945); they recommended formulation of purified IgG in 0.3 M glycine. The destabilizing affects of saline can be offset by the use of carbohydrates in the formulation of IgG (Back, Oakenfull, and Smith, 1979), however, at formulation pH < 6 carbohydrates do not improve the stability of native IgG in solution. It is interesting to note that saline found its way into formulation of immune serum globulin in the late 1950s with the practice of washing filters to improve IgG recovery.

The question of sterility has also influenced the formulation of IgG products. In the 1940s, the Oncley group recommended leaving IgG in the freeze-dried state in order to minimize risk from bacterial growth. Although sterile filtered solutions of IgG have been on the market for many years without clinical episodes from bacterial contamination, bacteria can grow in these solutions if introduced. For this reason, all bottles of liquid formulated IGIV should be visually inspected for evidence of bacterial growth in addition to filter testing of randomly removed bottles from each lot. Visual inspection, however, is dependent on the clarity of the solution and is of limited use if the turbidity is greater than 10 nephelometric turbidity units. Even though freeze-dried product is less likely to support bacterial growth, it is not less likely to be contaminated. Since contamination of liquid formulated IgG is more readily detected by visual inspection than is the case with freeze-dried products, there is a trade off of risk factors between liquid and freeze-dried product configurations.

Quality Assurance Testing

In addition to testing IGIV products for anticomplement activity and bacterial contamination, safety testing should be carried out for the presence of kallikren and prekallikren activator, which give rise to kinin-mediated hypotension. This is done by measuring the ability of the product to react with a synthetic substrate for kallikren,

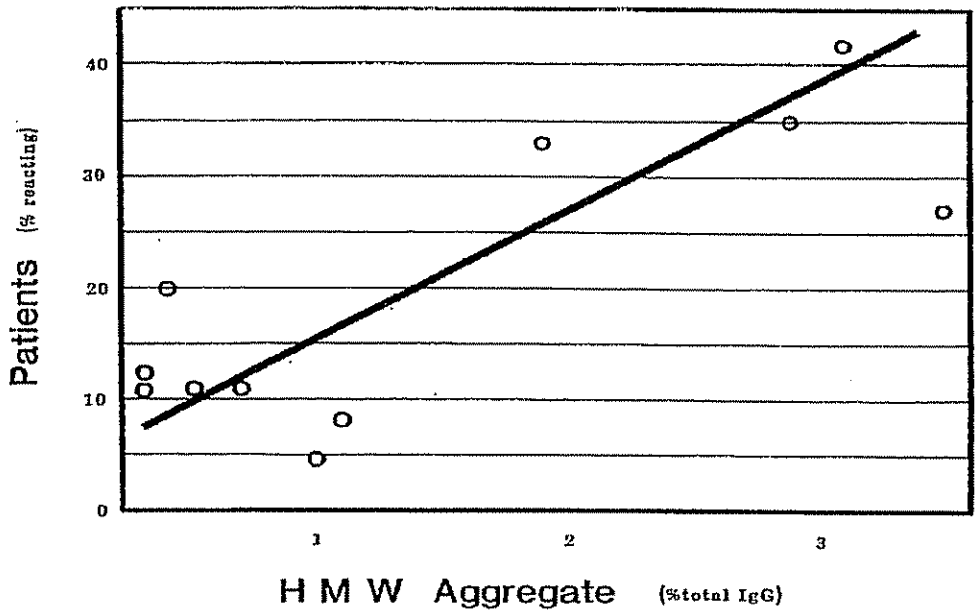

Fig. 5. Percent of patients reacting to various lots of reduced and alkylated human IgG, plotted against the amount of high-molecular-weight IgG aggregate found in each lot at the time of clinical use. No statistically significant correlations between product and patient reactions were found other than for percent of high molecular-weight aggregate ($p < 0.005$).

such as sodium benzoyl-L-arginine ethyl ester, in the presence and absence of added prekallikren. The measured activity should be compared and expressed relative to an acceptable reference sample. Safety testing in animals should also be done on final container samples from each lot. High dose peritoneal injection of guinea pigs will reveal the presence of gross hypotensive or hypertensive activity from the product, while low dose injection of rabbits will reveal the presence of pyrogens. Levels of antibodies to human red cell antigens should be measured using standard blood banking procedures and typing cells. Dilution titers of these antibodies should not exceed 1:8 (response of red cell agglutination assays to IgG product configurations make titers less than 1:8 not meaningful). Quantitative analysis of high-molecular-weight aggregates of IgG should be made by size exclusion HPLC, since small amounts (i.e., <3%) of this type of material can be clinically troublesome even in products that have been chemically modified to eliminate anticomplement activity (Figure 5).

The Final Product Container

One aspect of product formulation that has received little attention is the container in which the product is stored and sold. Studies with purified IgG in solution between

pH 6.6 and 7.2 have revealed that solution turbidity, amount of soluble aggregates, and the amount of protein precipitate were influenced by the composition of the inner wall of the containers in which the solutions were stored (McCue, 1980). Glass containers with greater negative surface charge were found to induce greater aggregation of the IgG, while plastic containers showed more complex trends, possibly the result of independent hydrophobic and ionic interactions with the surface of the containers.

FUTURE TRENDS

Among the many monoclonal antibodies being developed as therapeutic agents, those with specialized activity against malignant neoplasias have been under development longest. One such antibody well along in its development is L-6 (Oncogen, Inc., Seattle, WA, U.S.A.), which is now in Phase II human clinical trials. This antibody appears to carry out cell killing of malignant tissue by blocking the growth of the involved cells, an event known as "apoptosis," and not by complement-mediated cell destruction. Although apoptosis is the most common form of mammalian cell death, its role in antibody physiology has only recently been recognized (Trauth et al., 1989).

Another potentially useful aspect of antibodies is the ability to produce antigen-binding sites that act like enzymes (Baldwin and Schultz, 1989). Thus far, these abzymes have been produced that hydrolyze peptide bonds while maintaining high antibody specificity for the polypeptide of interest. Most recently a naturally occurring "abzyme" has been identified as a human autoantibody (Paul et al., 1989).

These newly identified physiologic roles of antibodies greatly enhance the potential of antibody therapy. It also means that the heterogeneity of antibody response must be looked at more closely when preparing monoclonal antibodies for therapeutic use.

Although the advent of the monoclonal antibodies has provided a method of producing large quantities of a specific antibody, it will be a long while, if ever, before it will be possible to create monoclonal antibody cocktails to mimic the normal polyclonal antibody response. Until that time it may well be that the plasma-derived polyclonal concentrates could serve as the ideal administration vehicle for monoclonals targetted at specific antigens (Rousell and Collins, 1989).

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